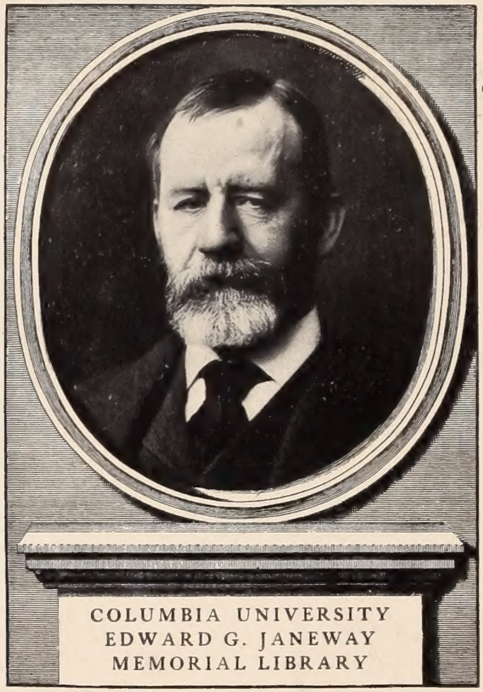
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THE
JOURNAL OF INFECTIOUS DISEASES

The
Journal of Infectious Diseases

Published by the Memorial Institute for Infectious Diseases

EDITED BY

LUDVIG HEKTOEN AND EDWIN O. JORDAN

IN CONJUNCTION WITH

FRANK BILLINGS F. G. NOVY

W. T. SEDGWICK

Volume 11

1912

Chicago, 1912

Composed and Printed By
The University of Chicago Press
Chicago, Illinois, U.S.A.

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NO. 1, JULY, 1912.

NO. 2, SEPTEMBER, 1912.

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ERRATA.

VOLUME 10.

P. 335, last line, "exhausted" should read "exhaustive."
P. 349, l. 16, "1 per cent" should read "2 per cent."
P. 366, l. 25, "Drs." should read "Dr."
P. 272, second footnote, expunge "9" following "N.S."
P. 275, l. 1, "ferment" should read "ferments."
P. 280, l. 2, "4 per cent" should read "2 per cent."
P. 282, l. 7, "saccharose, and salicin" should read "raffinose, and mannit."
P. 405, l. 31, "typical" should read "atypical."
P. 407, l. 3, "growth" should read "broth."

VOLUME 11.

P. 114, "B. paratyphosus "A" (University of Chicago)
" " "B" " " "

Table 3.

should read
"B. paratyphosus "A" (University of California)
" " "B" (University of California).

P. 122, l. 22, "papulum" should read "pabulum."
P. 123, l. 7, "1910, 7," should read "1910, 12,".

The

Journal of Infectious Diseases

PUBLISHED BY THE MEMORIAL INSTITUTE FOR INFECTIOUS DISEASES

VOL. II *July 1912* No. 1

A STUDY OF THE BACTERIOLOGICAL AND SANITARY CONDITION OF THE MILK SUPPLY OF NEW YORK CITY.*

M. C. SCHROEDER.

(*From the Research Laboratory, Department of Health, New York City.*)

The following study of the bacteriological and sanitary condition of the milk supply of New York City was undertaken at the request of Dr. W. H. Park, general director of the Division of Laboratories, and was carried on and completed under his direct supervision.

The milk samples which form the basis of this study were taken by the members of the bacteriological squad and comprised samples of both city and country milk. Owing to the distances from New York City at which the creameries were located and the difficulties of transportation these men had to be provided with the simplest kind of an outfit for taking these samples. This outfit consists of the following articles: an aluminium pipette 21 inches long and $\frac{1}{4}$ inch in diameter; a screw-necked vial 3 inches long and $\frac{7}{8}$ of an inch in diameter, with an aluminium cap bearing a serial number; a round tin box 5 inches in diameter and $3\frac{1}{4}$ inches high to contain the sample bottles; two sizes of copper pipette holders

* Received for publication April 5, 1912.

of the round type (see photograph), (*a*) holder 22 inches long, 2 inches in diameter, to hold 40 pipettes; (*b*) holder 22 inches long, 1 inch in diameter, to hold 10 pipettes. The pipettes, bottles, and cans are thoroughly sterilized on two consecutive days and then shipped once a week to the country inspectors.

The members of the bacteriological squad who take city samples carry pipettes, holders, and bottles of the same type, but in addition are provided with leather bags 18 inches long, log-cabin type, lined with aluminium and fitted with trays for holding the milk samples, and ice to keep them cold while being gathered.

Outfit carried by inspectors.

Method of taking samples in the country.—The samples are taken by means of the aluminium pipettes before described, placed in the one-ounce vials, packed in the round tins, placed in an old milk can, and thoroughly surrounded with ice. A hole is made in the bottom of the can so as to provide for drainage. The can is then wired, labeled, and shipped on the milk freight train together with the milk from which the samples were taken. Upon their arrival at the city terminal the samples are at once collected by an inspector who ices them again thoroughly and brings them to the laboratory, where he checks up the samples and writes his report. He also frequently takes control samples from the regular milk shipments. The method of taking the samples in the city is practically the same except that the inspectors use the bags described and bring the samples directly to the laboratory.

Methods employed at the laboratory.—(*a*) All glassware used in milk work is sterilized for an hour at 160° F on two consecutive days. The dilution bottles are of the square type and are gradu-

ated at 50 c.c. and 100 c.c., respectively. The water used for dilutions is ordinary tap water filtered through a Berkefeld filter and sterilized by boiling on two consecutive days in a specially constructed heater from which it is filled directly into the bottles and used without resterilizing. The pipettes used are of two kinds, the ordinary volumetric pipette graduated to 1/100 of a c.c. and a short pipette 5 inches long and $\frac{1}{4}$ inch in diameter with three markings, $\frac{1}{2}$ c.c.–1 c.c. (see Fig. 1). We are also using what we term the ring method.

FIG. 1.—Special pipette in use.

Ring method.—It was found after a number of experiments that a small $\frac{1}{8}$-inch No. 20 japanned curtain ring would take up within 5 per cent of 0.01 c.c. of milk and that by dropping this ring full of milk directly into the melted agar tube, we could eliminate one or more of the dilution bottles and thus save labor and a possible source of error. The rings are dipped into the milk by means of a platinum hook and dropped directly into about 8 c.c. of nutrient agar held in the tube.

Method of plating and counting.—Each inoculated tube is shaken 10 times and plated. The Petri dishes after marking are packed into paper-lined crates holding 60 or more plates and incubated at a temperature of 37° C. for 48 hours. They are then examined and counted and the count placed directly upon the plates. From these plates the counts are entered upon the bacteriological cards sent to us by the inspectors.

Method of recording data obtained from cards.—The items of information concerning the samples taken in the country are the location of the creamery, its owner, the name of the dairyman, the time the sample was taken, the temperature and the quality of the milk, the manner of cooling, number of samples, and the bacteriological result (see Fig. 2). The city card varies somewhat, as shown in the illustration (Fig. 3). In the laboratory the results from the creameries are recorded on the ledger sheet shown in Fig. 4; the

sheet gives the name of the owner, name and location of creameries controlled by him, the kinds of milk shipped, bacteriological counts, the temperature of samples, the manner of cooling the milk, the result of the dirt test, and other facts bearing upon the bacteriological results. These sheets are kept in ledger form and a monthly

FORM No. 86 F-1911 1738–11 (B) 1500

DEPARTMENT OF HEALTH

THE CITY OF NEW YORK

CREAMERY AT .... Average Count ....

Operator ....

Date .... 191 Air Temp. .... ° Fahr.

NAME	TIME	ICE	MILK	TEMP.	SAMPLE No	BACTERIAL COUNT

Signed .... Bacteriologist Signed .... Inspector

(Size of card, 5×8.)

FIG. 2.

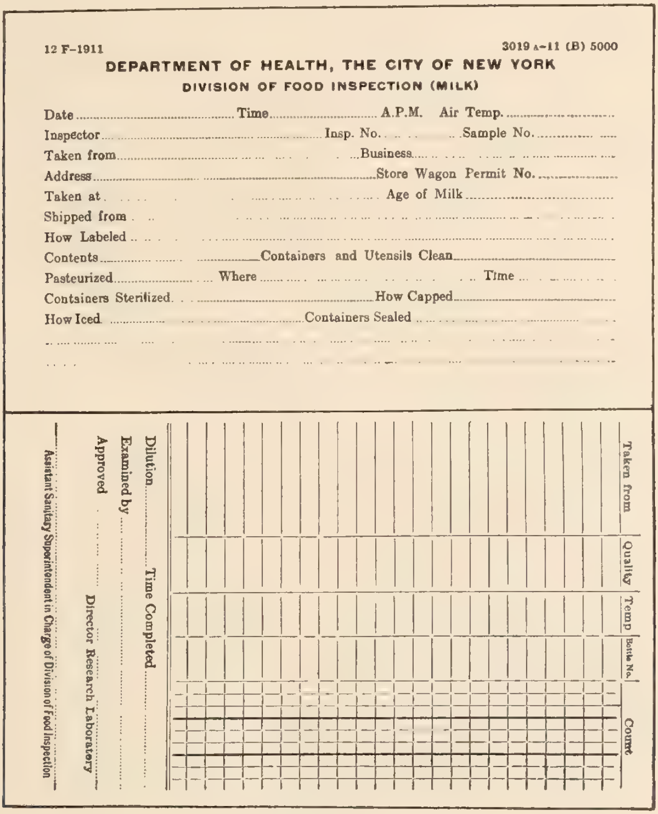
statement is drawn. The bacteriological results from the samples taken in the city are tabulated in a similar manner.

Analysis of samples.—The total number of bacteriological analyses made was 61,142. The various sources are best shown in Table 1.

TABLE 1.

Source	No. of Samples
Country creameries	22,761
Pasteurizing plants	5,049
Stores	19,876
Wagons	6,132
Hospitals	5,406
Infant health stations	504
Special examinations	1,414
	61,142

For the purpose of this study there were taken 20,334 samples of country milk and 1,950 samples of milk as delivered in the city. These samples of country milk represent the milk from 432 creameries situated as previously stated. The creameries are


12 F–1911 3019 A–11 (B) 5000

DEPARTMENT OF HEALTH, THE CITY OF NEW YORK

DIVISION OF FOOD INSPECTION (MILK)

Date .... Time .... A.P.M. Air Temp. ....
Inspector .... Insp. No. .... Sample No. ....
Taken from .... Business ....
Address .... Store Wagon Permit No. ....
Taken at .... Age of Milk ....
Shipped from ....
How Labeled ....
Contents .... Containers and Utensils Clean ....
Pasteurized .... Where .... Time ....
Containers Sterilized .... How Capped ....
How Iced .... Containers Sealed ....

Dilution .... Time Completed ....
Examined by ....
Approved .... Director Research Laboratory
.... Assistant Sanitary Superintendent in Charge of Division of Food Inspection

Taken from
Quality
Temp
Bottle No.
Count

(Size of card, 3×5.)

FIG. 3.

controlled by 120 of the large dealers who supply New York City with milk.

Analysis of country milk.—A study of the bacteriological findings as recorded in Table 2 shows that of the 20,334 samples taken for bacteriological examinations 7,756, or 38.14 per cent, contained less than 10,000 bacteria per c.c.; 6,633, or 32.61 per cent, contained from 10,000 to 50,000 bacteria per c.c.; 2,403, or

11.81 per cent, contained from 50,000 to 100,000 bacteria per c.c.; 2,723, or 13.39 per cent, contained from 100,000 to 1,000,000

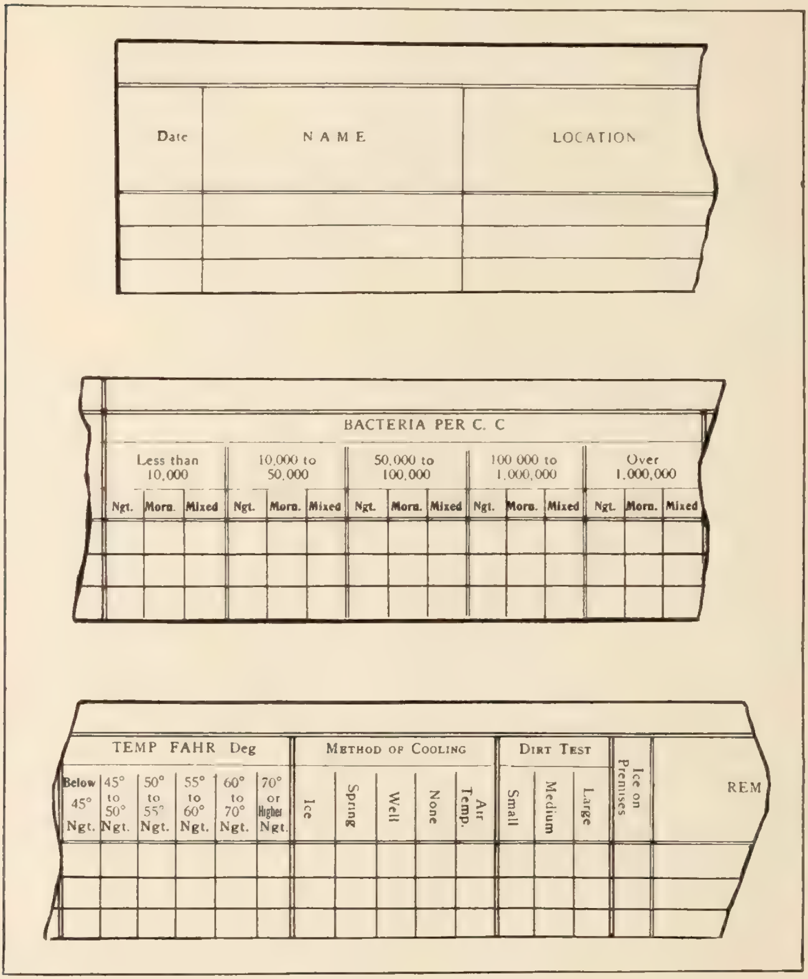


Fig. 4.—Headings on ledger sheet.

bacteria per c.c.; 819, or 4.02 per cent, contained 1,000,000 or over bacteria per c.c.

Adding together the number of samples which contained less than 50,000 bacteria per c.c. it will be found that 14,389, or 70.75 per cent of all samples analyzed, fall into this class.

Contrasting the highest with the lowest percentage of samples of each group it will be found that in the division of less than 10,000 per c.c. April ranks highest, having 673 samples, or 59 per cent, out of a total of 1,140 samples; August lowest, with 332 samples, or 23.8 per cent, out of a total of 1,393.

TABLE 2.

ANALYSIS OF BACTERIOLOGICAL COUNTS.

	Total of Samples	Bacteria per c.c.									
		Less than 10,000		10,000 to 50,000		50,000 to 100,000		100,000 to 1,000,000		1,000,000 or over	
		No.	Per-cent-age	No.	Per-cent-age	No.	Per-cent-age	No.	Per-cent-age	No.	Per-cent-age
January	1,274	579	45.44	472	37.	128	10.	91	7 14	4	.3
February	1,466	578	39.42	613	41.68	169	11.49	100	6.8	6	.4
March	1,241	682	54.90	421	33.89	90	7.24	39	3.13	9	.7
April	1,140	673	59.	289	25.14	78	6.78	94	8.17	6	.52
May	2,097	874	41.95	580	27.84	303	14.54	293	14.06	47	2.25
June	2,099	621	29.58	689	32.82	206	9.81	410	19.53	173	8.24
July	830	299	35.88	239	28.68	132	15.84	130	15.60	30	3.60
August	1,393	332	23.81	340	24.38	181	13.18	319	22.67	221	15.85
September	2,152	550	25.55	533	24.76	298	13.84	525	24.39	246	11.43
October	2,802	1,135	39.72	934	32.59	315	11.02	358	12.53	60	2.1
November	2,514	988	39.29	1,032	41 05	308	12.25	180	7.15	6	.23
December	1,326	445	33.55	491	37.02	195	14.71	184	13 87	11	.82
	20,334	7,756	38.14	6,633	32.61	2,403	11.81	2,723	13.39	819	4.02

In the 10,000 50,000 group February ranks highest, having 613 samples, or 41.68 per cent, out of a total of 1,466 samples; August lowest, with 340 samples, or 24.38 per cent, out of a total of 1,393.

In the 50,000–100,000 group July ranks highest, having 132 samples, or 15.84 per cent, out of a total of 830 samples; April lowest, with 78 samples, or 6.78 per cent, out of a total of 1,140.

In the 100,000–1,000,000 group September ranks highest, having 525 samples, or 24.39 per cent, out of a total of 2,152 samples; March lowest, with 39 samples, or 3.13 per cent, out of a total of 1,241.

In the group containing 1,000,000 or over per c.c., August ranks highest, having 221 samples, or 15.85 per cent, out of a total of 1,393 samples; November lowest, with 6 samples, or 0.23 per cent, out of a total of 2,514.

Temperature of milk at time of delivery.—The temperatures of the milk from which those samples were taken are shown in Table 3. Owing to the extreme haste with which these samples had to be taken at times, and other adverse circumstances, it was not always possible to procure the temperatures of the milk at the time it was sampled. We were fortunate enough, however, to obtain the temperature of 18,698 specimens of milk, and the percentages shown in Table 3 are based upon these figures. This table also shows the number and percentage of samples in the divisions adopted, namely, below 45° F., 45–50°, 50–55°, 55–60°, 60–70°, and 70° F. and over. They are as follows:

Below 45° F. there were 3,767 samples, or 20.1 per cent out of a total of 18,698.

45 to 50° F. there were 3,568 samples, or 19 per cent out of a total of 18,698.

50 to 55° F. there were 3,945 samples, or 21 per cent out of a total of 18,698.

55 to 60° F. there were 3,603 samples, or 19.2 per cent out of a total of 18,698.

60 to 70° F. there were 3,141 samples, or 16.7 per cent out of a total of 18,698.

70° F. or over there were 674 samples, or 3.6 per cent out of a total of 18,698.

Contrasting the highest with the lowest percentage of samples having a temperature of less than 50° F. it will be found that in the division of temperatures less than 50° F. February ranks highest, with 771 samples, or 72 per cent, out of a total 1,063 samples; August lowest, with 55 samples, or 4.6 per cent, out of a total 1,171.

In the division of temperatures from 50 to 60° F., June ranks highest, with 1,069 samples, or 54.8 per cent out of a total 1,969 samples; November lowest, with 570 samples, or 23 per cent out of a total of 2,438 samples.

In the division of temperatures from 60 to 70° F., August ranks highest, with 604 samples, or 51 per cent out of a total 1,171 samples; March lowest, with 34 samples, or 2.7 per cent out of a total of 1,241 samples.

TABLE 3.

TEMPERATURE OF MILK AS RECEIVED AT CREAMERIES.

Months 1911	Total Samples	Temperatures Stated in	Temperatures Not Stated in	Temperature Fahrenheit: Less than 45° No.	Less than 45° Percentage	45° to 50° No.	45° to 50° Percentage	50° to 55° No.	50° to 55° Percentage	55° to 60° No.	55° to 60° Percentage	60° to 70° No.	60° to 70° Percentage	70° and over No.	70° and over Percentage
January	1,274	987	287	408	41.33	269	27.23	179	18.1	80	8.1	42	4.25	9	0.9
February	1,466	1,063	403	521	49.01	250	23.51	123	11.57	82	7.7	47	4.42	40	3.75
March	1,241	1,224	17	397	32.46	449	36.6	251	20.5	86	7.	34	2.7	7	.57
April	1,140	1,024	116	315	30.71	337	32.8	225	21.9	91	8.8	37	3.6	19	1.8
May	2,097	2,034	63	105	5.16	265	13.02	441	21.68	625	30.72	549	26.49	49	2.40
June	2,099	1,969	130	27	1.37	121	6.1	533	27.06	536	27.22	615	31.23	137	6.95
July	830	721	109	15	1.98	31	4.2	137	19.	216	29.9	168	23.3	154	21.3
August	1,393	1,171	222	14	1.19	41	3.5	121	10.3	271	23.1	604	51.5	120	10.2
September	2,152	2,056	96	95	4.62	154	7.49	391	19.01	697	33.90	640	31.12	79	3.84
October	2,802	2,690	112	307	11.41	625	23.23	803	29.85	644	23.94	273	10.14	38	1.41
November	2,514	2,438	76	1,047	42.92	745	30.5	396	16.2	174	7.1	70	2.8	6	.24
December	1,326	1,321	5	516	39.	281	21.2	345	26.	101	7.6	62	4.6	16	1.2
	20,334	18,698	1,636	3,767	20.13	3,568	19.08	3,945	21.09	3,603	19.26	3,141	16.74	674	3.6

In the last division, 70° F. or over, July ranks highest with 154 samples, or 21 per cent out of a total of 721 samples.

Method of cooling.—The data which were gathered by the inspectors concerning the method of cooling employed by the dairymen to cool their milk are given in Table 4. It will be noticed

TABLE 4.

METHOD OF COOLING.

Month of Year	Number of Samples	Samples for Which Cooling Method Was Not Stated		Samples for Which Cooling Method Was Stated		Samples for Which Cooling Method Was Ice		Samples for Which Cooling Method Was Not Ice	
		No.	Percentage	No.	Percentage	No.	Percentage	No.	Percentage
January	1,274	454	35.63	820	64.35	422	51.46	398	48.53
February	1,466	851	58.04	615	41.95	371	60.32	244	39.67
March	1,241	290	23.36	951	76.63	449	47.21	502	52.78
April	1,140	69	6.05	1,071	93.94	659	61.53	412	38.47
May	2,097	237	11.47	1,860	88.53	1,345	72.31	515	27.69
June	2,099	635	30.22	1,464	69.74	812	55.46	652	44.53
July	830	130	15.66	700	84.33	354	50.57	346	49.42
August	1,393	224	16.08	1,169	83.91	583	49.87	586	50.12
September	2,152	358	16.62	1,794	83.31	525	28.71	1,269	71.23
October	2,802	242	8.63	2,560	91.34	621	24.25	1,939	75.74
November	2,514	263	10.46	2,251	89.36	499	21.95	1,752	77.83
December	1,326	106	8.25	1,220	91.50	268	21.94	952	78.03
	20,334	3,859	18.97	16,475	81.02	6,908	41.93	9,567	58.06

that the same discrepancy which has already been spoken of exists between the total number of samples taken and the number in which data were obtainable. The total number of samples in which the cooling method was stated was 16,475, or 81.02 per cent; the number in which the method of cooling was not stated was 3,859, or 18.97 per cent.

Of those in which the method of cooling was stated, ice was used in 6,908 instances, or 41.93 per cent, and was not used in 9,567 instances, or 58.06 per cent. We also found that the number and percentage of samples where ice was used varied in the different quarters of the year. This factor is also influenced by the character and location of the creameries, the dairymen drawing to the better-class creameries using ice more frequently than those who drew to the poorer class. Grouping the results obtained into the four quarters of the year gives us the following summary:

	Total Number of Samples Delivered at Creameries	Total Number in Which Cooling Was Stated	Number Using Ice	Percentage
1st quarter........	3,981	2,386	1,242	52
2d "	5,336	4,395	2,816	64
3d "	4,375	3,663	1,462	39
4th "	6,642	6,031	1,388	22
	20,334	16,475	6,908	41

It will be seen from these summaries how comparatively few dairymen use ice in cooling their milk. It is of interest to note that in July, 50 per cent of the milk brought in was cooled by ice, 61 per cent at a temperature of 50° F. or under, and 64 per cent of the milk contained less than 50,000 bacteria per c.c. The greater part of the samples taken in July were of those known as morning's milk. This milk is usually brought to the creameries without cooling, therefore the records of the temperatures of the milk in July were high and the bacteriological counts low.

The preceding analysis was based upon the total number of samples taken and their relation to each other as a whole. In the following analysis (Table 5) the milk is divided into the three grades in which it is delivered to the creameries by the dairymen; namely, night's, morning's, and mixed milk. It therefore shows in addition the number of samples of each grade taken, together with the number and percentage of samples falling into each one of the five groups of bacteriological counts. It also shows the temperatures of the samples taken from these three grades of milk as they were delivered at the creameries, and the number and percentage of the samples in each of the six divisions we have adopted as a standard of temperatures. It also gives a detailed statement of the methods of cooling employed by the dairymen. Taking up the different groups of bacteriological counts we find that the morning's milk had the highest percentage of low counts, 82.19 per cent having less than 50,000 bacteria per c.c., and the night's milk had the lowest percentage of samples in that group, namely, 64.8 per cent. A brief summary of these percentages is given on p. 12.

Night's milk.—Considering the preceding summary it will be noticed that while 64 per cent of the samples are under 50,000, 34

per cent contained 50,000 or over per c.c., notwithstanding the fact that the largest number of samples were taken during the cold months, when the atmospheric temperature would naturally have some effect in reducing the bacterial content and holding bacterial growth in check. This comparatively large percentage of bacteria may be ascribed to either one or both of two factors: increased contamination of the milk due to the fact that the cows are housed and not kept as clean as in the summer time, and use of utensils which were not clean.

BACTERIAL COUNTS PER C.C.

Type of Milk	Total Number of Samples	Less than 50,000		50,000 to 100,000		100,000 to 1,000,000		1,000,000 to 100,000,000	
		Number of Samples	Percentage	Number of Samples	Percentage	Number of Samples	Percentage	Number of Samples	Percentage
Night's	9,885	6,477	64.8	1,244	12.45	1,622	16.2	542	6.4
Morning's...	1,202	988	82.19	111	9.23	89	7 4	14	1.6
Mixed	9,247	6,924	74	1,048	11 5	1,012	11.11	263	2.8
		14,389	70.76	2,403	11.81	2,723	13.39	819	4.02

While comparatively few samples of morning's milk were sent to the laboratory for analysis, it is of interest to note that 46.8 per cent had less than 10,000 bacteria per c.c. and 82 per cent of the samples contained less than 50,000 bacteria per c.c. This low bacterial content held its own during the months of April, May, June, and July, when the percentages were respectively 81, 75, 79, 87 per cent of the total number of samples taken of morning's milk for those months. These results bear out the statement made above concerning time as a factor as well as the extra contamination of milk during the winter months, and the effect of the unclean utensils. Most of the morning's milk was delivered within two hours after milking. The night's milk and the mixed milk, being made up of night's and morning's milk, is, on account of its being held, more readily affected than either of the two other grades by the three factors stated; namely, by increased contamination due to the unclean condition of the cattle, unclean utensils, and lack of icing. Owing to the fact that it approximates closely the grade of milk delivered to city consumers for the

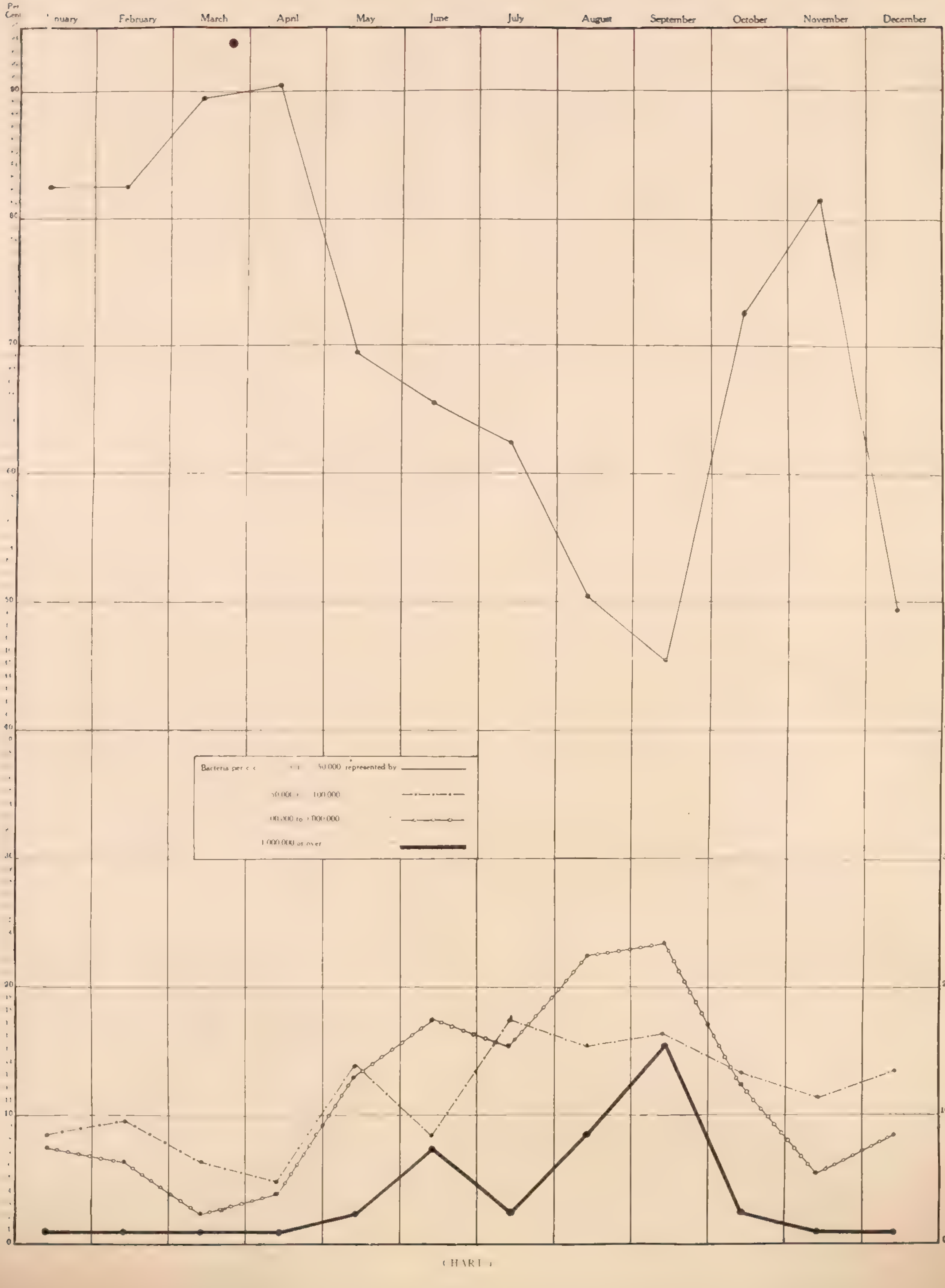

Per Cent
February
March
April
May
June
July
August
September
October
November
December
90
80
70
60
50
40
30
20
10
0
Bacteria per c.c. 50,000 represented by
100,000
1,000,000 or over

CHART 1

entire year, a detailed study of the bacterial count of the mixed milk was made. The bacteriological curve obtained is shown in Chart 1.

A comparison of the chart with the table gives us the percentages shown. These are briefly summarized as follows:

Months	Total Number of Samples of Mixed Milk		Less than 50,000 Bacteria per c.c.		50,000 to 100,000 Bacteria per c.c.		100,000 to 1,000,000 Bacteria per c.c.		1,000,000 or over Bacteria per c.c.	
	Number of Samples	Percentage	Number of Samples	Percentage	Number of Samples	Percentage	Number of Samples	Percentage	Number of Samples	Percentage
January	912	100	757	82.96	81	8.87	72	7.89	2	.10
February	961	"	796	82.83	93	9.67	66	6.86	6	.62
March	900	"	810	90.00	60	6.6	27	2.9	3	3.3
April	467	"	423	90.57	22	4.7	21	4.49	1	.21
May	1,257	"	877	69.68	175	13.82	172	13.58	33	2.6
June	931	"	613	65.73	84	8.98	166	17.76	68	7.27
July	429	"	268	62.47	83	17.33	68	15.64	10	2.33
August	385	"	202	52.46	60	15.	88	22.	35	8.75
September	518	"	234	45.17	84	4.37	120	23.39	80	15.59
October	761	"	544	71.48	101	13.23	95	12.44	21	2.75
November	1,223	"	1,010	82.5	138	11.17	74	5.99	1	.08
December	503	"	390	77.53	67	13.32	43	8.54	3	.59

It will be observed that the percentage of milk containing less than 50,000 bacteria per c.c. drops steadily from its highest level of 91 per cent in April until it reaches the lowest point, or 45 per cent, in September, and that the percentage of milk containing a million or more bacteria per c.c. follows the inverse curve and reaches its highest point in July. Table 5 also shows clearly that most of the samples were brought in at a temperature ranging from 50 to 60° F., and a considerable portion between 60 and 70° F.

Having thus briefly considered the bacteriological content of the milk as received at the creameries together with a few of the factors influencing the same, it was thought best to consider also the milk as it was offered for sale in New York City, in order to ascertain: (1) what was the general bacteriological content of the milk sold; (2) the age of milk as offered for sale; (3) the relative bacteriological content of the raw milk sold in cans or bottles; of the pasteurized milk sold in cans or bottles, together with samples taken of the milk pasteurized by the flash process and known as heated milk; (4) some of the sanitary conditions surrounding its care and sale. For

this purpose 1,950 samples were selected from the large number of samples examined. Four months of the year, January, April, July, and October, were selected as representative types of the different seasons, and 100 samples of each month's supply of milk of the grades mentioned were chosen, except in the case of heated milk where it was impossible to procure this number from different dealers. These numbers represent the milk supplied by 160 of the principal dealers, being carefully selected to represent as many sources and dealers as possible. The data procured are set forth in Table 6. Considering these data in detail, we find that the raw milk sold in cans and bottles taken as a whole gives the following results:

Months	Total Number of Samples	Less than 50,000 Bacteria per c.c.		50,000 to 100,000 Bacteria per c.c.		100,000 to 1,000,000 Bacteria per c.c.		1,000,000 or over Bacteria per c.c.	
		Number of Samples	Percentage	Number of Samples	Percentage	Number of Samples	Percentage	Number of Samples	Percentage
January	200	57	28.5	22	11.	78	40.53	43	20.5
April...	200	47	23.5	33	16.5	88	44.	30	18.
July ...	200	25	12.5	11	5.5	94	47.	70	35.
October	200	28	14.	20	10.	80	40.	72	36.

Contrasting the above results with the bacteriological counts of the milk as delivered to the creameries in the country during these months, the following differences present themselves:

Months	Total Number of Samples	Less than 50,000 Bacteria per c.c.		50,000 to 100,000 Bacteria per c.c.		100,000 to 1,000,000 Bacteria per c.c.		1,000,000 Bacteria per c.c.	
		Number of Samples	Percentage	Number of Samples	Percentage	Number of Samples	Percentage	Number of Samples	Percentage
January									
Country.....	1,274	1,051	82.4	128	10	91	7.13	4	.3
City........	200	57	28.5	22	10.5	78	39.	43	21.5
April									
Country.....	1,140	962	84.3	78	6.7	94	8.1	6	0.52
City........	200	47	23.5	33	10.5	84	42.0	36	18.
July									
Country.....	830	538	64.	132	15.84	130	15.60	30	3.6
City........	200	25	12.5	11	5.5	94	47.	70	35.
October									
Country.....	2,802	2,069	73.84	315	11.02	358	12.5	60	2.1
City........	200	28	14.	20	10.	80	40.	72	36.

The increase in the bacterial content of city milk over that of country milk is marked, and not wholly accounted for by the factor of time in allowing increase of bacteria. This may be partly accounted for by the fact that milk was frequently shipped in cans and bottles which were not clean and were poorly iced in transit.

Age of milk.—The following figures concerning the age of milk as sold in New York City are based upon the date placed upon the labels attached to the cans. These dates do not, however, give the true age of the milk, as in numerous instances the milk is 12 or more hours old before it reaches the creamery, and then it may be held over at the creamery from 12 to 24 hours longer, before it is placed upon the milk train for transmission to the city.

Months	Total Number of Samples	Number Age Not Stated	Number Age Stated	24–36 Hours Percentage	36–48 Hours Percentage	48 Hours and over Percentage
January	200	62	138	72	19.	7.2
April	200	6	194	72	13.6	13.9
July	200	30	170	93	....	15.
October	200	12	188	88	25.	16.

Based upon the above figures it can be safely stated that 78 per cent of the raw milk reaches New York and is sold from 24 to 36 hours after leaving the creamery, 10 per cent, 36 to 48 hours, 9 per cent, 48 hours and over, some of it being more than 72 hours old. The actual age would be about an additional 12 to 24 hours.

Temperature of raw milk as offered for sale in New York City.—The temperature of the milk while offered for sale as shown by the following data is interesting:

TEMPERATURES.

Months	Total Number of Samples	Under 45° F.		45–50° F.		50–55° F.		55–60° F.		60–70° F.	
		Number of Samples	Percentage	Number of Samples	Percentage	Number of Samples	Percentage	Number of Samples	Percentage	Number of Samples	Percentage
January	200	171	85.5	14	7.	15	7.5	..	..	..	..
April	200	157	78.5	28	14.	15	7.5	..	..	..	..
July	200	82	41.	65	32.5	38	19.	11	5.5	4	2.
October	200	109	54.5	70	35.	21	10.5	..	..	..	..
		519	64.8	177	22.1	89	11.1	11	1.37	4	0.5

Taking the total number of samples of raw milk selected for study, it will be seen that 64.8 per cent of the milk examined was held in New York at a temperature of less than 45° F.; 22 per cent was found to be from 45 to 50° F.; 11 per cent was from 50 to 55° F.; 1.37 per cent from 55 to 60° F.; and 0.5 per cent 70° F. and over.

Comparison of bacterial counts of raw milk as sold in cans and bottles. The figures upon which this comparison is based are given in Table 6. Taking the average bacterial count of the samples for each month, we find that with the exception of the samples examined in October there is not the great difference in the counts we had hoped to find in favor of bottled milk. A comparison of these counts is given below:

Months	Containers	Average Bacterial Counts
January	Cans	318,500
	Bottles	317,450
April	Cans	295,450
	Bottles	169,050
July	Cans	510,000
	Bottles	289,000
October	Cans	380,160
	Bottles	43,230

Comparison showing the variation of the quantities of milk held at a temperature below 45° F.:

	January	April	July	October
	Percentage	Percentage	Percentage	Percentage
Cans	90	89	71	89
Bottles	95	96	76	90

It will be seen from the foregoing that the advantage of lower bacterial count and lower temperatures is slightly in favor of the milk sold in bottles.

Pasteurized milk sold in cans.—Pasteurized milk sold in cans is delivered as a rule to the hospitals, and therefore the output is fairly limited. It, however, represents the type of milk used by the hospitals during the past year.

PASTEURIZED MILK IN CANS.

PERCENTAGE OF AGE OF MILK.

Months	Not Stated	Stated	24–36 Hours	36–48 Hours	48 Hours
January	10	90	62	23	15
April	33	67	34	54	12
July	79	21	81	19	..
October	72	28	18	78	4

From the above it will be noticed that in the month of July only 21 per cent of the milk had the age stated on the tag.

PERCENTAGE OF TEMPERATURES.

Months	Below 45° F.	45–50° F.	50–55° F.	55–60° F.	60–70° F.
January	92	8	..	..	..
April	41	50	9	..	..
July	23	28	39	5	5
October	87	5	8	..	..

PERCENTAGE OF BACTERIOLOGICAL COUNTS.

Months	Less than 50,000 Bacteria per c.c.	50,000 to 100,000 Bacteria per c.c.	100,000 to 1,000,000 Bacteria per c.c.	1,000,000 and over Bacteria per c.c.
January	84	5	10	1
April	88	4	7	1
July	68	12	17	3
October	83	11	3	3

PASTEURIZED MILK IN BOTTLES.

PERCENTAGE OF AGE OF MILK.

Months	Not Stated	Stated	24–36 Hours	36–48 Hours	48 Hours and over
January	16	84	47	27	26
April	6	94	40	40	20
July	50	50	14	54	32
October	67	33	24	67	9

PERCENTAGE OF TEMPERATURES.

Months	Under 45° F.	45–50° F.	50–55° F.	55–60° F.	60–70° F.
January	90	..	..	4	6
April	62	24	14	.	..
July	37	19	30	14	..
October	62	21	17	..	..

PERCENTAGE OF BACTERIOLOGICAL COUNTS.

Months	Under 50,000 Bacteria per c.c.	50,000 to 100,000 Bacteria per c.c.	100,000 to 1,000,000 Bacteria per c.c.	1,000,000 and over Bacteria per c.c.
January	77	19	4	.
April	66	10	13	5
July	49	20	31	..
October	64	12	22	2

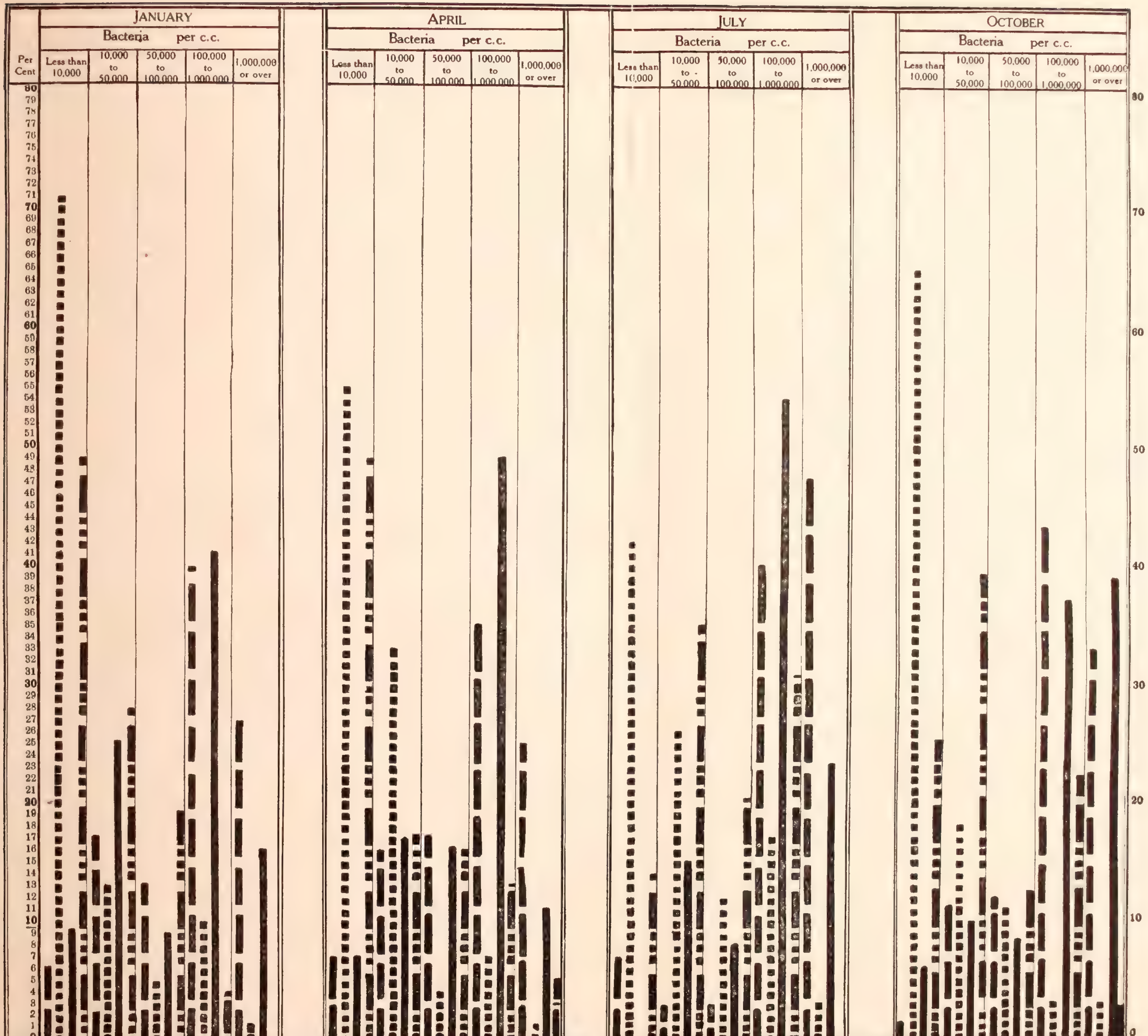
From the preceding tabulation it is seen that the bacterial counts in pasteurized milk are considerably less than in raw milk. Four factors, acting singly or together, account for the considerable percentage of persisting high counts: (1) improper pasteurization; (2) contamination of the milk through careless handling or unclean utensils; (3) holding too long, even up to 36 hours after pasteurization; (4) improper cooling methods.

The comparative bacteriological values of the four grades of milk sold in New York City is graphically shown in Chart 2, and accompanies the data given below.

Months	Type of Milk	Bacteriological Counts				
		Less than 10,000 Bacteria per c.c.	10,000 to 50,000 Bacteria per c.c.	50,000 to 100,000 Bacteria per c.c.	100,000 to 1,000,000 Bacteria per c.c.	1,000,000 and over Bacteria per c.c.
		Percentage	Percentage	Percentage	Percentage	Percentage
January	Raw milk in cans	6	17	13	37	27
	Past. " "	71	13	5	10	1
	Raw " " bottles	9	25	9	41	16
	Past. " "	49	28	1	10	4
April	Raw milk in cans	7	16	17	35	25
	Past. " "	55	33	4	7	1
	Raw " " bottles	7	17	16	49	11
	Past. " "	49	17	16	13	5
July	Raw milk in cans	7	3	3	40	47
	Past. " "	42	26	12	17	3
	Raw " " bottles	..	15	8	54	23
	Past. " "	14	35	20	31	..
October	Raw milk in cans	1	11	12	43	33
	Past. " "	65	18	11	3	3
	Raw " " bottles	6	10	8	37	39
	Past. " "	25	39	12	22	2

Heated milk in bottles.—As previously stated, the term heated milk is a name applied to milk which has been pasteurized by the flash process. This type of pasteurization is gradually being replaced by the "holder process" as the latter has been found more satisfactory. The numbers therefore are smaller than those of the raw milk, or those of the milk pasteurized by the holder process. The results are grouped together in tables on p. 19. The conditions shown in this table give fairly accurately the irregular results of the flash method of pasteurization.

Heated milk in cans.—As before stated, the milk known as heated milk is milk which has been pasteurized by the flash process.



KEY { Raw Milk in Cans represented by
" " " Bottles " "
Pasteurized Milk in Cans represented by
" " " Bottles " "

CHART 2.

This process was gradually replaced during the year by the holder process and we have, therefore, only 100 samples for the entire year. The results obtained are shown in Table 6.

HEATED MILK IN BOTTLES.

PERCENTAGE OF AGE OF MILK.

Months		Not Stated	Stated	24–36 Hours	36–48 Hours	48 Hours and over
January	100 samples	52	48	62	25	13
April	50 "	38	62	26	45	29
July	50 "	62	38	..	100	..
October	50 "	46	54	15	85	..

PERCENTAGE OF TEMPERATURES.

Months	Under 45° F.	45–50° F.	50–55° F.	55–60° F.	60–70° F.
January	77	21	2	..	..
April	52	14	22	12	..
July	12	50	32	6	..
October	66	24	18	..	..

PERCENTAGE OF BACTERIOLOGICAL COUNTS.

Months	Less than 10,000 Bacteria per c.c.	10,000 to 50,000 Bacteria per c.c.	50,000 to 100,000 Bacteria per c.c.	100,000 to 1,000,000 Bacteria per c.c.	1,000,000 and over Bacteria per c.c.
January	16	29	27	23	5
April	10	32	16	40	2
July	18	50	14	18	..
October	20	36	16	28	..

Summary.—The conclusions based upon the preceding analysis may be summarized as follows:

1. The greater portion of the milk is delivered by the dairymen to the creameries with a low bacterial content, namely, less than 50,000 bacteria per c.c. Out of 20,334 samples, 14,389 were below the figure given.

2. The greater portion of milk sold in New York City as raw milk, whether in cans or bottles, contains from 50,000 to 1,000,000 bacteria per c.c. and a considerable percentage contains over 1,000,000 bacteria per c.c.

3. The pasteurization of milk by the holding process, though still leaving much to be desired, reduces greatly the number of bacteria, besides destroying any pathogenic varieties.

4. Ice is not sufficiently used to cool the milk.

5. The average temperatures of the milk as delivered to the creameries by the dairymen, with the exception of the winter months, is still too high for milk to be shipped to New York, and has an unfavorable influence upon the milk by aiding the growth of bacteria.

6. Greater care should be observed to obtain clean milk by sterilizing the cans and bottles.

7. The greater part of the milk sold is from 36 to 48 hours old, but a considerable portion is from 72 to 96 hours old, and some even more than 96 hours. This is true of both raw and pasteurized milk.

In closing, thanks are due to the former and present members of the bacteriological squad for the hardships undergone and the careful interest displayed in their work. Thanks are also due to the Misses Hazel and Helen Connors, and Messrs. J. J. O'Brien, E. J. McCullen, and T. Schroeder, for their share in the compilation of the data required for this study.

THE ROCKFORD (ILL.) TYPHOID EPIDEMIC.*

Edwin O. Jordan and Ernest E. Irons.

The city of Rockford is situated on Rock River in the northern part of the state of Illinois. The population is estimated at about 50,000 (1900, 31,051; 1910, 45,401). In January and February, 1912, a sudden outbreak of typhoid fever occurred, involving about 200 bed cases. The writers were requested by the city authorities on February 14 to investigate and report on the cause of the epidemic.[1]

Record-cards were prepared, and first-hand data were obtained in the case of every patient of whom it was possible to learn. At the time of our inquiry typhoid fever was not a reportable disease in Rockford, but through the active co-operation of the Rockford physicians and with the assistance of the city authorities and the daily press, what is believed to be a very complete list of the actual bed cases was soon obtained. We were fortunate, through the courtesy of Dr. D. W. Day of the department of hygiene of the public schools, in securing the services of several very efficient school nurses for collecting the data regarding the typhoid fever cases. The knowledge of local conditions thus placed at our disposal greatly facilitated the investigation.

In all, a record of 199 cases occurring between January 23 and February 29, 1912, was obtained. In 177 of these cases definite information was secured regarding absence from the city prior to the outbreak. Of this number 173 had not been away from Rockford for at least 30 days prior to the onset of their illness. The cases comprised 102 males and 97 females. The age distribution is as follows:

Years		Years	
0– 5	13	31–45	28
6–15	46	45+	18
16–30	88	Not noted	6

* Received for publication May 21, 1912.

[1] We were ably assisted in our investigation by Mr. A. H. Hixson.

The approximate date of the earliest symptoms was determined in 187 cases. Where exact information could not be obtained, the date of the physician's first visit could generally be ascertained with accuracy. Hill's experience[1] has shown that in the neighboring state of Minnesota seven days probably represents the average interval between the physician's first visit and the earliest symptoms, and our own experience tends to confirm this view. Tabulating the results in three-day periods according to this method the following table is obtained:

January 23–25	1	February 7– 9	31	February 21–23	1
January 26–28	5	February 10–12	24	February 24–26	1
January 29–31	26	February 13–15	15	March 1–28	9
February 1– 3	41	February 16–18	6		
February 4– 6	37	February 18–20	1		

The date of appearance of the earliest symptoms places the date of typhoid infection for the majority of the cases in the week following January 16. As will appear presently, this coincides exactly with the other facts bearing on the origin of the epidemic.

The dates of "taking to bed" seem to give a less precise indication of the probable date of infection. Arranged in similar three-day periods, the dates of taking to bed are as follows:

January 23–25	1	February 7– 9	30	February 21–23	12
January 26–28	1	February 10–12	28	February 24–26	1
January 29–31	1	February 13–15	37	March 1–28	11
February 1– 3	15	February 16–18	13		
February 4– 6	22	February 18–20	18		

In a number of cases the record-cards show that the doctor was called before the patient had gone to bed, in others that the patient had been in bed for a week or more before summoning a physician. An aid to fixing the precise date of infection is the important circumstance, to be discussed presently, that the typhoid epidemic was ushered in by an outbreak of diarrhea which affected over 70 per cent of all those who afterwards developed typhoid fever. It is perhaps partly owing to this diarrheal outbreak that there was a large proportion of typhoid patients who kept their feet for a considerable time after first feeling ill, attributing their symptoms to

[1] "The Mankato Typhoid Fever Epidemic," *Jour. Infect. Dis.*, 1911, 9, p. 435.

the after-effects of the "intestinal grip" from which they had previously suffered. In many individual instances of which we learned there was a history of "intestinal grip January 16–18; patient has not been well since." It therefore seemed probable that in the Rockford outbreak the date of onset was more accurately placed by the method of dating back seven days from the physician's first visit than by the method of reckoning from the date of taking to bed.

There were in all 24 deaths from typhoid fever[1] attributable to the outbreak, two deaths occurring in January (January 6 and 27) not being included in our tabulation. Placing the date of infection in the week following January 16, the deaths occurred in the following order:

Third week after the initial infection	3	deaths.
Fourth " " " " "	4	"
Fifth " " " " "	5	"
Sixth " " " " "	6	"
Seventh " " " " "	3	"
Eighth " " " " "	1	"
Ninth " " " " "	1	"
Eleventh " " " " "	1	"

The deaths distributed according to age and sex are as follows:

Sex	Age	Cases	Deaths	Percentage
Males	0–19	33	1	3.1
Females	0–19	47	6	13.0
Males	20+	69	9	13.0
Females	20+	50	7	14.0
	Not known	..	1	....
Total		199	24	12.1

While the numbers dealt with are too small to have by themselves much statistical value, the results are of interest from their substantial agreement with the grouping observed by Hill in the similarly caused Mankato epidemic,[2] save that the proportion of female deaths under 20 years was much smaller in his series. The discrepancy between the male adult and the male child case mortality in Rockford is as marked as in the series reported by Hill (11.1 per cent males 20+; 3.6 per cent males 0–19).

[1] Two deaths occurring before February 1 were attributed to the "gastro-intestinal grip."

[2] Loc. cit.

PRIMARY AND SECONDARY CASES.

Owing to the fact that the period of infection probably extended over at least several days, it is impossible in the present outbreak to make a perfectly sharp distinction between primary and secondary cases. There were in all:

170	1-case families
13	2-case "
1	3-case family

In four of the 2-case families the second case developed 15–20 days after the first case and late in the outbreak (February 16, February 17, February 22, and March 13, respectively). These four cases are probably to be regarded as secondary infections due to contact. Early in the outbreak measures were taken to guard against the spread of infection. With the co-operation of the newspapers and city health authorities wide publicity was given to dangers of transmission of infection from existing cases and to the necessary precautions to be observed. Nurses, physicians, and those in close attendance on the sick very generally received anti-typhoid vaccination, and the intercourse between sick and well was reduced to a minimum. It is perhaps partly due to such measures as well as to the absence of fly infection and other hot-weather influences that the rather sudden cessation of the epidemic was due. Whatever the explanation, the number of secondary cases seemed to be less than usual. Only 11 cases developed in the month of March; three of these had suffered from enteritis January 16–20.

In an explosive outbreak of typhoid fever occurring in winter, fly-borne infection and contact infection may be excluded as possible causes. The information obtained by our house-to-house canvass likewise excluded milk-supply as a primary factor. As many as 49 different milk dealers supplied the families in which typhoid fever had developed and in no instance was the number of cases on any particular milk-route disproportionate to the number of customers on that route.

Many circumstances pointed to the city water supply as the source of infection. Although about one-twentieth of the population is said to use water from private wells, many of which are

obviously badly placed, there was no evidence that these wells were responsible for the outbreak. On the contrary the users of well water were notably freer from disease than the users of city water. Several instances came to our notice of families using private wells, all the members of which were exempt save those that drank city water at school or at their places of business. In every one of the 178 cases of typhoid fever regarding which information could be obtained the city water was used constantly or occasionally.

The events preceding the outbreak of typhoid fever were indicative of water infection. On January 16–20 there had been a severe and sudden outbreak of gastro-enteritis or epidemic diarrhea. This was explosive in character and bore all the marks of a general water infection. On January 19, one of the Rockford morning papers printed a column and a half under the caption "Epidemic of Intestinal Grip Sweeps Rockford," in the course of which the statement was made that "*The* * was told last night [January 18] by doctors that they have been kept busy night and day for two days past and there is no apparent let-up." This is the first published statement we have been able to find and seems to fix the beginning of the outbreak on January 16–17. This conclusion was confirmed by the results of an intensive investigation made by us to which reference will be made presently.

The epidemic of enteritis was widespread and affected persons of every age and social station. Although no part of the city was free from the disease, certain districts suffered more severely than others. A probable reason for the unequal distribution of cases will appear later.

The proportion affected in factories with and without private wells was particularly significant. In three large factories employing about 1,150 persons we obtained on inquiry a history of 77 who had been attacked by enteritis; in another group of six factories situated in the same district as the foregoing there was a history of 433 attacks in an employed population of 617. The sole discoverable difference was that the first group of factories had private deep wells (5.8 per cent of force attacked by enteritis), while the second group of factories was supplied only with city water (70.2 per cent attacked by enteritis). Since many of those

employed in the factories lived in homes supplied with city water it is to be expected that there would be some cases among the employees of the relatively exempt factories.

The incidence of the enteritis upon the users of city water is shown also by the following among a large number of similar instances collected by us.

1. Family A., 5 members, all drinking well water at home: C. A., 9 years old, attending B school, where he drank city water, was attacked with enteritis; none of others affected. T. A., head of family employed at N. factory where 80 per cent suffered from enteritis. T. A. never drank much at factory and after first cases appeared carried water from home.

2. Family O., living outside city limits and using own well. M. O. worked at N. factory and drank water there: he was affected; his wife W. O. was in Rockford on January 19 and developed enteritis; the other members of the family, three in number, were not affected. All the members of two neighboring families, none of whom were in the city January 16–20, remained well.

3. Family K., eight members, city water; M. K., head of family, employed in factory with private well, never drank water at home. All members of family except M. K. sick with enteritis.

Such instances showed conclusively the connection between the city water supply and the outbreak of gastro-enteritis. Indeed the season of year at which the epidemic occurred, its explosive nature, its general distribution, the number of persons affected, and its general resemblance to similar outbreaks elsewhere traced to water-borne infection pointed unmistakably in this direction, even before the exact facts were ascertained.

There were all grades of severity exhibited in the cases, from exceedingly virulent choleraic symptoms to a slight indisposition, with mild diarrhea. Two persons are said to have died as the direct result of the enteritis. Many mild cases on the other hand never came under a physician's care. We were fortunate in securing a satisfactory basis for an approximate estimation of the number of cases. At our request the school nurses visited all public schools, and, by consulting the records of absence and interviewing the children with the co-operation of the teachers of the various grade

rooms, were able to obtain a reasonably accurate list of all school children that suffered from enteritis on January 16–20. In the case of some of the youngest children definite information could not be obtained, so that the actual number of cases of illness of which we obtained record is unquestionably somewhat below the truth. In a school enrolment of 6,303 at the time of the outbreak we learned definitely of at least 1,357 cases of enteritis. Inquiry from physicians and others showed that school children had not suffered disproportionately to the other age-groups of the Rockford population. If we assume that the same ratio of attack holds for all ages as for the school population, then there were not less than one-fifth, or about 10,000 of the total population, affected.

Some estimates of local observers are much higher than this. One of the daily papers on January 19 declares that "The universality of the disease is apparent in the fact that nearly every business institution has its victims from two to a score or more, and almost every block and every house has some member suffering from an intestinal difficulty." Some factories were forced to shut down on account of the large number of employees affected. "Public schools were decimated" on January 19. "Superintendent P. R. Wallace found this morning that every school had a large number of pupils on the absent list." One writer in a morning paper (February 13) asserts that "probably three out of four people in Rockford were affected" by the "intestinal grip" epidemic.

An intensive investigation of a limited area was made by us of one block in South Rockford (bounded by Main, Church, Morgan, and Loomis Streets). Of the 46 persons resident in this block, 40 suffered from gastro-intestinal disturbance. These were all users of the city water and drank of it during the period January 16–20. Of the six remaining persons one later developed typhoid fever and two drank no city water or drank it only in small amounts. The earliest date of onset (where the dates could be exactly stated) was the evening and night of January 16, when four individuals became ill. These were followed by five on January 17, five on January 18, one on January 19. Twenty-two could not recall the exact day of illness. The usual symptoms were nausea, vomiting, and diarrhea, followed by extreme prostration. Fever was

rarely present. Abdominal pain and tenderness were present in some cases. In some instances, diarrhea was the only symptom, and in two nausea and vomiting without diarrhea. Recurrences of the enteritis at intervals of three to four days were noted in nine cases.

It is thus clear that two extensive outbreaks of disease occurred in Rockford in January and February, 1912, one a more or less violent gastro-enteritis with a short period of incubation, the other a typical epidemic of typhoid fever following the gastro-enteritis after about two weeks. As is well known, similar outbreaks in Mankato, Minn., and other localities have been traced to water infection. All indications, therefore, pointed to an infection of the public water supply on or about January 16.

It is important to consider here the prevalence of typhoid fever in Rockford prior to 1912. The following table gives the record of deaths so far as obtainable.

	1906	1907	1908	1909	1910	1911	1912
January		1	0	0	1	0	2
February		0	0	0	0	0	6
March		0	0	0	1	0	15
April		0	0	0	0	0	2
May		1	0	0	0	2	
June		1	0	0	0	3	
July		0	0	0	3	0	..
August		0	0	0	0	0	.
September		0	1	0	4	1	
October		[illegible]	3	0	0	0	.
November		0	0	2	0	0	
December		0	0	0	1	2	..
Total	1	3	4	2	10	8	.

Unfortunately, typhoid fever cases have not been generally reported in Rockford and the number of deaths is too small to warrant any general conclusions. At the same time the fact is worth noting that there were as many deaths from typhoid fever reported in 1910 as in the four years, 1906–9 together. So, too, is the fact that the 1910 death-rate from typhoid fever in Rockford was over 22, a rate so high as to be ordinarily associated with a more or less polluted water supply. We have further gained the impression in conversations with Rockford physicians that scattered cases of enteritis or "intestinal grip" have been more common in Rockford in recent years than is quite normal for a community

with a water supply that is absolutely uncontaminated at all times.

On the other hand, a number of careful analyses of the city water made by the State Water Survey under the direction of Professor Bartow at various times both before and after the diarrheal outbreak invariably showed a water of great purity. If contamination had occurred, therefore, it must have been occasional, not continuous.

WATER SUPPLY.

The following description of the water supply is taken in part from personal inspection, in part from a special "Report on the Enlargement and Extension of the Water Supply and Distribution System of the City of Rockford, Ill.," made by Messrs. Alvord, Maury, and Mead in November, 1910.

The original waterworks system was constructed in 1874-75, when the population of the city was about 12,000. It is stated that the present site was selected because of the occurrence there of excellent springs. "The spring supply was developed by laying tile drains through the gravel in the immediate vicinity of the pumping station, and to a so-called filter well. Above the tile drains, where they entered the filter chamber, were placed coke, gravel, and stone, and the water entering the tile flowed to the well, and, passing upward through the filtering material into the filter chamber and then to the pumping pit, was finally taken by the pumps. A pipe also connected the upper portion of the filter well with the river, so that the river water might be admitted on emergency. The actual flow of the original springs is unknown, nor is there any record of the capacity of the springs when developed as above described."

Some years later the filter well was pronounced inadequate to supply the demand for water and a new well 38 feet in depth was sunk south of the pumping station which drew its supply from the extensive sand and gravel deposits in that section of the city. "Throughout this period the river water was frequently admitted into the mains for fire purposes." In 1885 analysis of the well water was said to show that it was inferior in quality to the river

water and the well was hence abandoned, and only river water used. The need of a different supply was soon recognized, and the first artesian well was sunk to the Potsdam sandstone to a depth of 1,530 feet. Additional wells were drilled in the years immediately following.

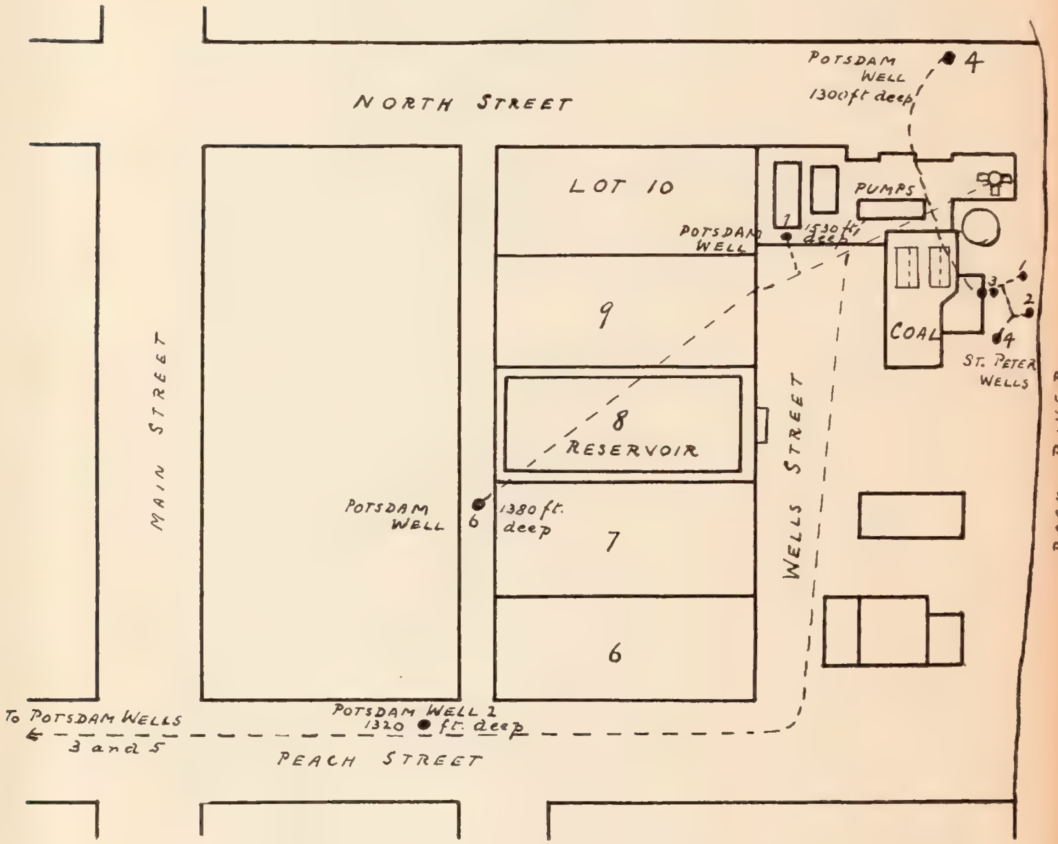


FIG. 1.—Rockford pumping station and surroundings.

At present the water supply of Rockford is obtained from a group of eleven wells, five to the St. Peter and six to the Potsdam sandstone. The St. Peter wells and three of the Potsdam wells are connected by the tunnel system with the suctions of the centrifugal pumps at the bottom of the shafts. The other three

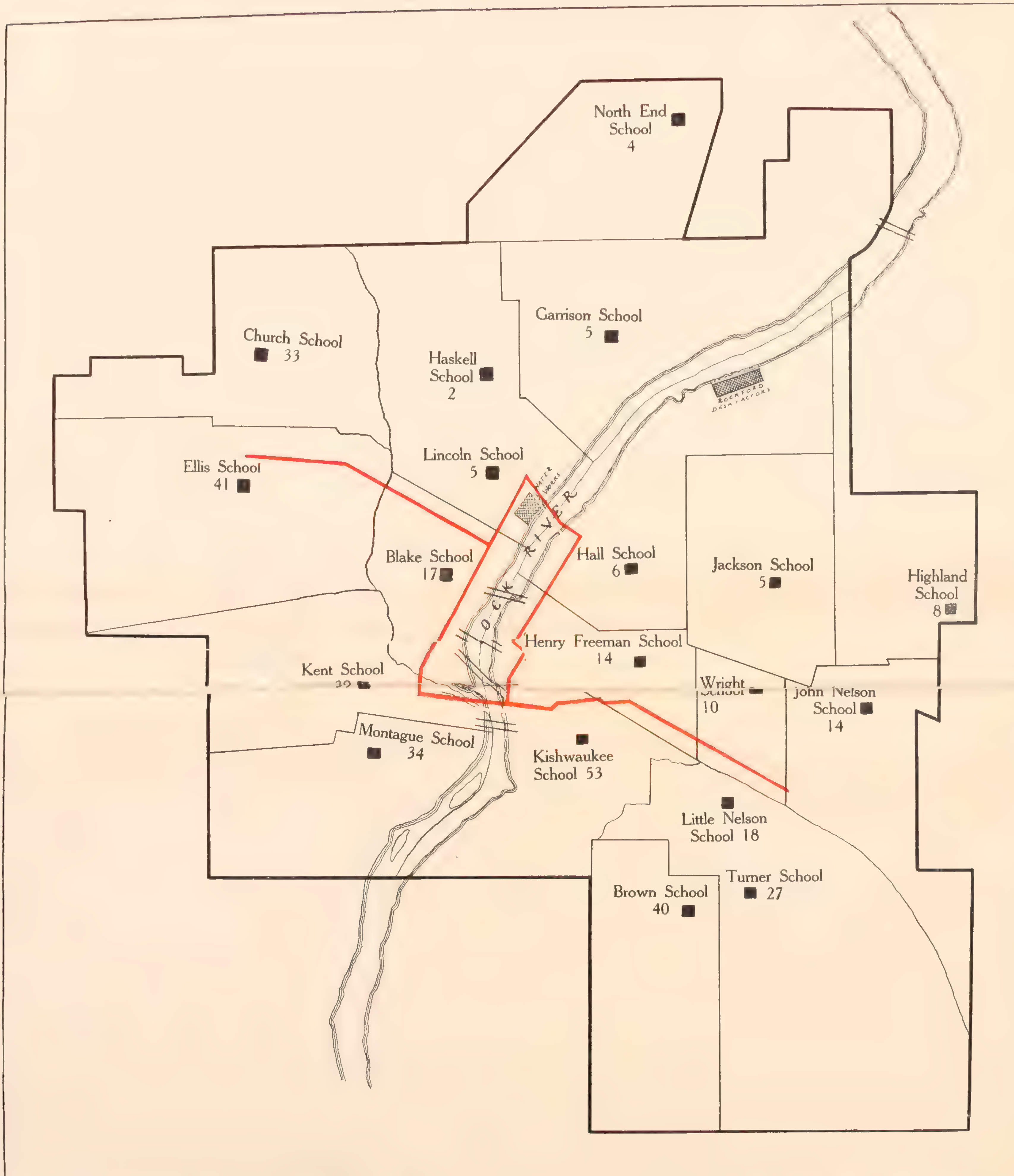


FIG. 2.—The figures show the percentage of children in each school who suffered from enteritis. The red lines represent the chief water mains. The main draught of water is south from the pumping station along the west bank of Rock River (factory district). The land is high to the north and east of the pumping station and the water pressure correspondingly poor.

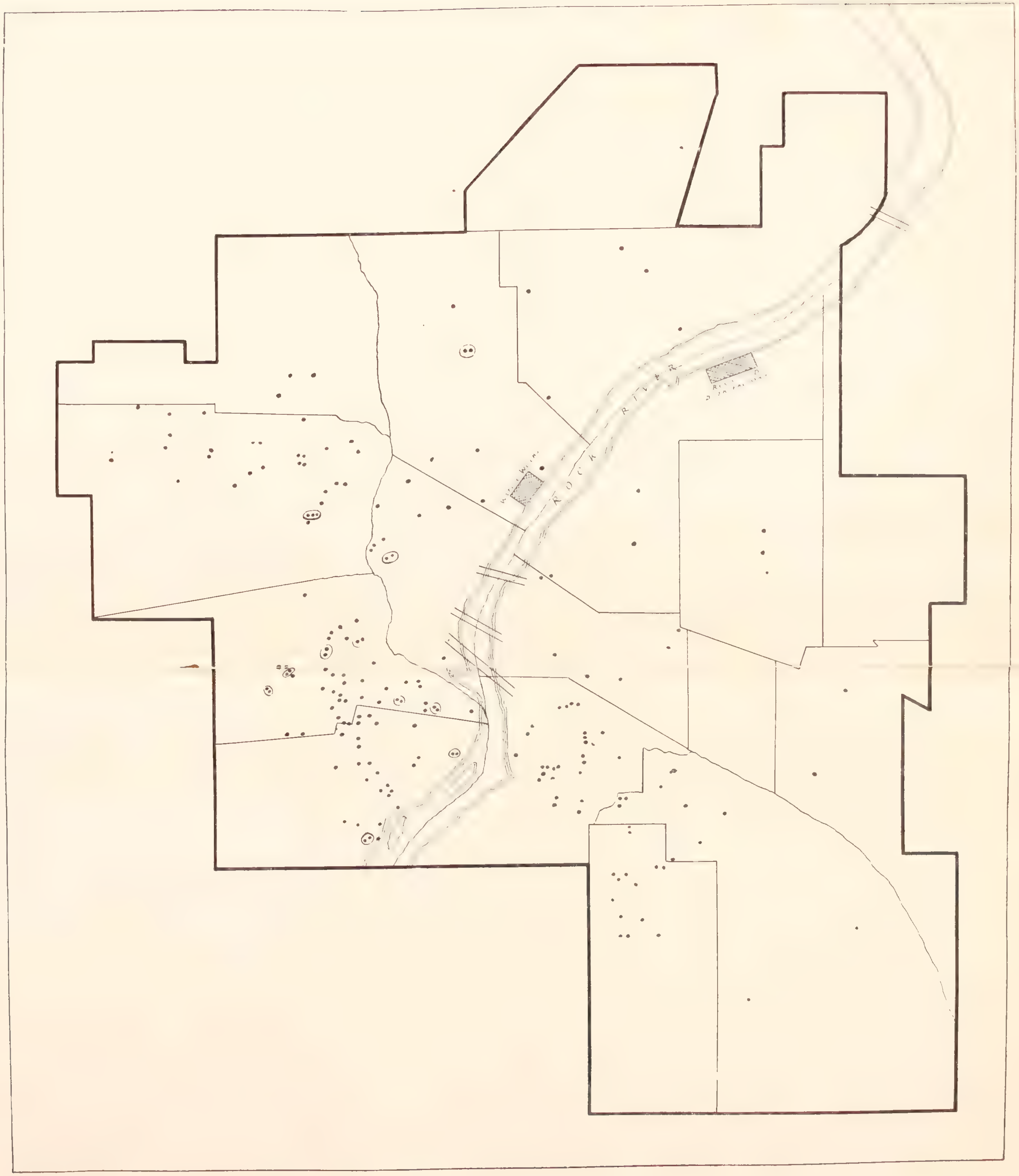


FIG. 3.—Spot map showing distribution of typhoid cases in Rockford epidemic. Note correspondence with distribution of enteritis cases (Fig. 2).

Potsdam wells (Nos. 2, 3, and 5) are not joined to the tunnel system, but are connected with a common discharge main through which the water flows by gravity to the pumping station (Fig. 1).

In 1892–93 a concrete storage reservoir of about 1,250,000 gallons capacity was constructed about 80 feet southwest of the pumping station. The reservoir is filled during hours of minimum pumping and drawn upon when the draft is heavier than the wells can supply. Ordinarily the draft upon the reservoir begins about 7 A.M. when the factories open, and continues till two or three o'clock in the afternoon, at which time the reservoir begins to be replenished. Under usual conditions about 200,000 to 300,000 gallons are drawn from the reservoir each day between 7 A.M. and 2 P.M. and a corresponding amount restored to it before the following morning. This supply is entirely inadequate and methods of extension are being considered.

From this brief description it is evident that the Rockford waterworks is not thoroughly adapted to the present use. Originally planned to furnish spring water, it has with relatively slight changes been used to distribute river water and again later artesian well water.

Examination of the present conditions showed several dangerous possibilities. At the time of the enteritis outbreak it seems to have been at first generally believed in Rockford that river water had been turned into the mains through the pipe connecting the upper portion of the older filter well with the river. On Monday night, January 15–16, there had been a large fire at the Rockford Desk Factory (Fig. 2) and a consequent heavy draft on the water system. A number of years ago it was the custom to admit river water to the mains for fire service, but this does not appear to have been done in any instances in recent years. All waterworks officials, including responsible engineers and assistants at the pumping station, denied the use of river water at the time in question. Three days after the fire one observer (see *Morning Star*, January 20) noted that the box near the old filter well containing the wheel and rod for the river valve was covered with snow and ice and showed no signs of having been disturbed for a long period (Fig. 4). Besides such direct evidence against the admission

of river water it may be pointed out that the distribution of the enteritis and typhoid fever cases was not what would have been expected had there been a rush of contaminated river water through the mains toward that section of the city where the fire was in progress. On the contrary, the northeast section of Rockford where the fire occurred suffered less severely than some other sections (see Figs. 2 and 3).

FIG. 4.—Southeast corner of pumping station, showing location of old filter well and valve location. Rock River east of station.

In addition to the pipe between the river and the main at the pumping station, there are some other river water pipes in connection with the fire protection systems at various factories along the river bank. Visits were made to a number of these factories and information obtained regarding the number of employees, number of enteritis cases, and other data. No record could be found of any fire in these factories on or about January 16 and there was no evidence of any sort, either in the distribution of cases or in the specific factory conditions, that river water had been admitted into the mains from this source. Samples of city water obtained by us from taps in various parts of the factory district showed no evidence of contamination.

Since everything pointed to some unusual occurrence at the time of or shortly following the Desk Factory fire which gave rise to an unusual infection of the public water supply, the whole water system was subjected to careful inspection. Three undoubtedly dangerous conditions were brought to light in the course of our inquiry. These may be considered separately.

1. *Peach Street well No. 2.* -This is one of the Potsdam wells lying southwest of the reservoir (Fig. 1). It was sunk in 1886 and is 1,320 feet in depth. The water from the deep well is raised by an air lift pump out of the well tube where it strikes a cast iron umbrella and drops back into a pit about eight feet deep which opens by a manhole to the street. The bottom of the pit is about 13 feet below the street level. When the well is being used the water flows by gravity from this pit to the suction well, by means of an overflow pipe placed about two feet from the bottom. At the time of our inspection of the pit, holes in the cover of the manhole permitted free access of street drainage to the pit. The pit was nearly full of water at the time of our inspection. Besides allowing entrance of surface water, the walls of the pit are not impervious and allow water from the surrounding soil to enter. A brick was noticed to be missing from the pit wall at one point. It is thus apparent that whatever the condition of the water from well No. 2 when drawn from the depths, it becomes mixed with the contaminated water of the pit before passing to the pumping station. In point of fact all samples collected from the trough running from this pit to the pumps have shown marked bacterial contamination, as indicated by a colony count averaging several thousand and the presence of *B. coli* in all 1 c.c. samples.

Whether the use of water from this contaminated well pit had anything to do with the explosive outbreak of January 16 may perhaps be determined by consideration of the following facts: The water from well No. 2 was allowed to run into the pumping pit on only one date between the summer of 1911 and the end of January, 1912. That date was Tuesday, January 16. On that day, according to the records at the pumping station, the air compressor in well No. 2 was operated from 11:45 A.M. to 10 P.M. Reference to the dates of enteritis attacks given above will show that

the date of onset of the earliest cases was the evening and night of January 16. This corresponds exactly with what would be expected if the explosion of enteritis and the subsequent outbreak of typhoid fever were due to the introduction on January 16 of the undoubtedly contaminated water of well No. 2 into the water system.

A further significant occurrence at this time was the filling of the storage reservoir which had been depleted to an unusual extent by the demand for water caused by the Desk Factory fire on January 15. During the hours when No. 2 well was being pumped, the reservoir water level was raised from seven feet to its usual height of about 18 feet; in all 804,600 gallons were pumped into the reservoir on January 16 as compared with 229,800 on January 15, and 251,550 on January 17. Since the reservoir is filled from the mains, part of the large volume of water entering the reservoir on January 16 must have been derived from well No. 2. This body of water was not completely removed from the reservoir for several days, the amount of water taken out of the reservoir and replaced by pure water daily being about 250,000 gallons, and it seems possible that contaminated water entered the mains from the reservoir for several days after the original infection had ceased. This may explain the dragging out of the enteritis cases over a number of days. It is not possible of course to determine how long the contamination persisted. Not only was the contaminated water diluted from day to day with the pure water, but there is reason to suppose that, as is usually the case under such circumstances, dangerous bacteria did not multiply, but died out more or less rapidly.

2. *The reservoir.*—As already stated, the capacity of the reservoir is only about one-third to one-fourth of the total daily supply. In times of heavy demand water is drawn from both the shaft system and reservoir; and in times of light demand, by means of a regulating valve at the old filter well, the water pumped by the shaft system in excess of the demand is stored in the reservoir. The bottom of the reservoir is below ground level and below the usual level of the river, 180 feet distant. On the north side of the reservoir the ground level is somewhat above the usual high water level in the reservoir (Fig. 5).

Ordinarily the height of water in the reservoir is said to range between 18.7 and 14.0 feet, but the level is not infrequently lowered considerably below 14 feet. Since the reservoir is partly below the ground level it became important to determine whether ground water could enter when the reservoir level is reduced to a degree reached in practical operation. On January 16, as already mentioned, the reservoir level was brought down to 6.6 feet.

FIG. 5.—Top of reservoir seen from north.

Examination of water samples taken by us from the reservoir when the level was over 14 feet had shown no evidence of contamination. When, however, the level was reduced to 6.6 feet on February 14 and kept there for about twelve hours, bacterial evidence of pollution was found. Inspection of the walls also showed some seepage. At one point along a small crack soft mud was found ridged out toward the interior of the reservoir. The same crack when examined immediately after the reservoir was emptied had shown no indication of inward seepage. The bacterial pollution was most marked on the north side of the baffle wall which runs at a height of about six feet longitudinally through the reservoir from east to west, separating the basins into north and south

divisions. The surroundings of the reservoir are not prepossessing (Figs. 5, 6, 7, and 8). Owing to the proximity of the sewage-polluted Rock River, whose level is sometimes above the bottom of the reservoir, to the nearness of the Peach Street sewer, and to other sources of contamination, the soil and ground water in the neighborhood of the reservoir and pumping station must, as a rule, contain dangerous impurities. One privy is in use within five feet of the north wall (Fig. 8). The reservoir levels on January 15 and 16 are recorded as follows:

January 15, 1912	7 A.M.	18′7″	
	9	16′7″	
	12 Noon	15′5″	
	3 P.M.	15′5″	
	4	15′7″	
	5	15′7″	
	6	15′5″	
	7	16′	
	8	16′6″	
	9	17′2″	
	10	17′9″	
	11	18′5″	Fire started at 11:45 P.M.
January 16, 1912	12 Midnight	18′6″	
	1 A.M.	17′3″	
	2	16′	
	3	15′2″	
	4	13′8″	
	5	12′4″	
	6	11′1″	
	7	8′7″	
	8	7′1″	
	9	6′6″	
	11:45 A.M.	....	Well No. 2 started into the pumping pit
	12 Noon	7′1″	
	2 P.M.	8′	
	3	8′9″	
	4	9′9″	
	5	10′8″	
	6	11′4″	
	7	12′6″	
	8	13′6″	
	9	14′9″	
	10	16′2″	Well No. 2 discontinued.
	11	17′4″	
	12 Midnight	18′7″	

TABLE 5

Month During Which Samples Were Taken	Total Number of Patrons from Whom Samples Were Taken	Total of Samples: Night	Morn.	Mixed	Total	Bacteria per Cubic Centimeter — Less than 10,000: Night No.	Per-centage	Morn. No.	Per cent age	Mixed No.	Per cent age	10,000 to 50,000: Night No.	Per cent-age	Morn No.	Per cent-age	Mixed No.	Per cent age	50,000 to 100,000: Night No.	Per cent age	Morn No.	Per cent age	Mixed No.	Per-centage	100,000 to 1,000,000: Night No.	Per cent age	Morn No.	Per cent age	Mixed No.	Per cent age	1,000,000 or over: Night No.	Per-cent-age	Morn No.	Per cent age	Mixed No.	Per cent age
January	[illegible]	[illegible]	[illegible]	612	1,274	29	[illegible]	142	47.17	408	[illegible]	9	11.75	114	7.8	549	58.25	16	26.22	31	10.9	81	8.57	6	9.73	13	4.31	22	7.56	1	1.65	1	[illegible]	2	[illegible]
February	61	[illegible]	[illegible]	661	1,466	105	34.4	125	62.50	348	56.19	106	34.75	59	29.50	448	46.59	67	21.96	9	1.5	93	9.67	7	8.8	7	3.5	66	6.86					6	.62
March	[illegible]	324	17	900	1,241	152	46.9	8	47.05	522	57.9	125	30.5	[illegible]	29.4	288	31.99	28	8.64	2	11.1	60	6.6	[illegible]	3.08	2	11.1	22	2.9	6	1.55			3	[illegible]
April	842	[illegible]	[illegible]	467	1,149	29	51.26	19	51.35	328	[illegible]	155	[illegible]	11	29.72	93	20.55	52	8.17	1	12.8	22	4.7	[illegible]	11	[illegible]	8.1	21	4.49	[illegible]	.8			1	.1
May	[illegible]	803	7	1,25[illegible]	[illegible]	53	[illegible]	14	[illegible]	[illegible]	[illegible]	214	26.65	14	57.7	552	27.8	123	15.31	5	[illegible]	175	13.82	[illegible]	14.6	[illegible]	8.1	[illegible]	[illegible]	[illegible]	1.61	1	[illegible]	[illegible]	[illegible]
June	[illegible]	1,11[illegible]	19	[illegible]	[illegible]	[illegible]	[illegible]	20	[illegible]	[illegible]	[illegible]	26	29.1	19	55.70	544	30.50	118	10.54	4	8.16	84	8.95	[illegible]	21.55	[illegible]	10.20	106	[illegible]	14	9.25	1	[illegible]	68	[illegible]
July	[illegible]	1	[illegible]	[illegible]	[illegible]	30	25	[illegible]	11.11	[illegible]	[illegible]	57	20.75	21	[illegible]	101	[illegible]	30	10.50	13	6.91	83	17.[illegible]	[illegible]	24.88	9	4.78	68	[illegible]	18	5.45	[illegible]	1.66	10	[illegible]
August	[illegible]	937	1	385	[illegible]	251	[illegible]	23	32.39	108	[illegible]	[illegible]	[illegible]	[illegible]	52.50	94	[illegible]	111	11.84	10	11.05	60	15	[illegible]	25.79	8	11.26	88	[illegible]	[illegible]	13.10	[illegible]	9.85	[illegible]	[illegible]
September	[illegible]	1,552	8	518	2,13[illegible]	60	18.12	[illegible]	[illegible]	127	24.76	309	18.70	[illegible]	[illegible]	107	20.80	204	13.14	10	12.19	84	16.37	[illegible]	25.38	11	13.41	160	[illegible]	165	10.6	1	1.21	80	15.59
October	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	42.50	[illegible]	10.60	306	40.86	[illegible]	[illegible]	45	53.53	245	31.17	197	10.31	17	12.77	101	[illegible]	[illegible]	12.93	10	12.03	97	[illegible]	59	[illegible]			21	2.78
November	[illegible]	[illegible]	57	[illegible]	[illegible]	453	[illegible]	19	33.33	510	41.79	[illegible]	11.71	[illegible]	10.35	494	10.91	166	13.45	4	7.01	158	11.17	15	7.69	11	10.29	41	[illegible]	5	.40			1	.08
December	[illegible]	[illegible]	51	503	[illegible]	248	51.51	13	[illegible]	[illegible]	[illegible]	[illegible]	54.21	14	15.10	200	[illegible]	126	13.78	2	[illegible]	67	13.32	[illegible]	17.67	1	[illegible]	15	[illegible]	[illegible]	.88	1	[illegible]	3	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	34.54	[illegible]	46.[illegible]	3,808	41.18	3,082	31.18	[illegible]	36.19	3,116	[illegible]	1,244	12.67	111	9.23	1,048	[illegible]	[illegible]	63.00	89	7.40	1,012	[illegible]	712	5.18	11	1.16	[illegible]	[illegible]

TABLE 5—*Continued*

Month During Which Samples Were Taken	Temperatures Fahrenheit* — Below 45° F.: Night No.	Per-cent age	Morn No.	Per cent age	Mixed No.	Per-cent-age	45° to 50°: Night No.	Per-cent-age	Morn No.	Per-cent-age	Mixed No.	Per-cent-age	50° to 55°: Night No.	Per-cent-age	Morn No.	Per cent age	Mixed No.	Per cent age	55° to 60°: Night No.	Per-cent-age	Morn No.	Per-cent-age	Mixed No.	Per-cent-age	60° to 70°: Night No.	Per-cent-age	Morn. No.	Per-cent-age	Mixed No.	Per-cent-age	70° or over: Night No.	Per-cent-age	Morn No.	Per-cent-age	Mixed No.	Per-cent-age	Method of Cooling: Samples from Dairymen Using Ice	Samples from Dairymen Not Using Ice	Samples from Dairymen Using Springs or Wells	Cooling Method Not Indicated	Total of Samples Taken
January	1	[illegible]	[illegible]	8.26	[illegible]	51.76	21	[illegible]	19	29.24	196	[illegible]			79	32.64	150	14.5			43	17.76	37	5.56			42	17.35					9	[illegible]			[illegible]	398		[illegible]	1,274
February	[illegible]	[illegible]	56	1.11	[illegible]	47.25	[illegible]	[illegible]	17	[illegible]	[illegible]	[illegible]	16	6.6	21	11.4	56	13.5	[illegible]	[illegible]	49	26.6	31	13.5			51	[illegible]	16	[illegible]			40	21.72			[illegible]	244		[illegible]	1,466
March	11	43.82	1	7.14	[illegible]	[illegible]	121	[illegible]	6	[illegible]	[illegible]	[illegible]	13	[illegible]	3	21.4	265	23.12	17	5.24	2	[illegible]	67	7.55	11	[illegible]	1	7.14	32	[illegible]			1	7.4	6	.67	449	[illegible]		290	1,241
April	[illegible]	42.40	1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	1	[illegible]	119	[illegible]	89	14.21	[illegible]	8.10	[illegible]	36.44	235	[illegible]	1	10.80	52	14.24	18	[illegible]	9	[illegible]	16	[illegible]			19	[illegible]			659	409	3	[illegible]	[illegible]
May	[illegible]	[illegible]	1	[illegible]	[illegible]	2.82	105	[illegible]			160	[illegible]	180	22.32	1	2.7	200	21.58	[illegible]	27.90	14	[illegible]	386	[illegible]	215	26.60	7	[illegible]	327	27.14	8	[illegible]	14	[illegible]	27	2.24	[illegible]	[illegible]		[illegible]	2,097
June	18	[illegible]	[illegible]	[illegible]	[illegible]	.21	[illegible]	[illegible]			54	[illegible]	230	24.13			294	[illegible]	70	27.27			260	28.40	360	36.36			[illegible]	[illegible]	[illegible]	[illegible]	42	[illegible]	60	6.42	812	647	[illegible]	635	[illegible]
July	11	[illegible]			1	1.19	[illegible]	[illegible]			15	1.17	47	23.73			[illegible]	[illegible]	52	26.26			104	18.97	67	[illegible]	45	25.89	76	16.69	5	[illegible]	[illegible]	[illegible]	6	1.79	[illegible]	[illegible]	10	[illegible]	830
August	1	1.56			1	[illegible]	57	1.44			4	[illegible]	120	12			21	8.1	206	21.72	1	1.4	64	[illegible]	434	[illegible]	10	14	160	[illegible]	1	4.8	60	84.	26	7.40	583	574	112	[illegible]	[illegible]
September	[illegible]	[illegible]			5	10	106	6.99			48	9.81	296	19.53			97	19.40	502	[illegible]	1	1.51	194	39.75	500	[illegible]	11	21.21	126	[illegible]	8	[illegible]	[illegible]	77.26	20	4.09	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
October	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	.71	[illegible]	[illegible]			45	[illegible]	[illegible]	32.24	1	[illegible]	[illegible]	20	[illegible]	17.73	5	6.13	295	42.57	68	[illegible]	40	49.20	[illegible]	[illegible]			33	40.59	[illegible]	[illegible]	621	[illegible]	[illegible]	[illegible]	2,802
November	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	29.46	15	[illegible]	[illegible]	6	307	[illegible]	102	8.46	8	[illegible]	[illegible]	[illegible]	64	[illegible]	16	[illegible]	100	8.30			17	[illegible]	53	4.39			3	[illegible]	[illegible]	[illegible]	499	[illegible]		[illegible]	[illegible]
December	[illegible]	[illegible]	1	[illegible]	165	33.60	180	[illegible]	1	[illegible]	100	[illegible]	198	24.94	[illegible]	[illegible]	112	[illegible]	50	6.30	5	10.10	[illegible]	[illegible]	12	[illegible]	10	32.20	[illegible]	8	3	[illegible]	9	[illegible]	4	[illegible]	268	[illegible]	1	[illegible]	1,326
	2,190	23.14	62	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	77	7.33	1,663	20.31	[illegible]	[illegible]	121	11.52	[illegible]	23.12	1,704	18.62	140	[illegible]	[illegible]	20.82	1,675	17.69	226	[illegible]	1,240	15.12	[illegible]	1.04	424	[illegible]	[illegible]	[illegible]	[illegible]	9,219	348	3,859	20,334

* Percentages based on number of samples in which temperature was stated. See Table 3

TABLE 6

Quarterly Statement	Number of Samples Taken for Examination	Number of Dealers from Whom Samples Were Taken	Number of Creameries Represented	Unknown Sources	Origin of Milk as Stated on Labels		Age of Milk: Stated		Age of Milk: Not Stated		Age of Milk: From 24 to 36 Hours		Age of Milk: From 36 to 48 Hours		Age of Milk: 48 Hours and over		Method of Cooling: Stated		Method of Cooling: Not Stated		Temperatures: Under 45° F.		Temperatures: From 45° to 50° F.		Temperatures: From 50° to 55° F.		Temperatures: From 55° to 60° F.		Temperatures: From 60° to 70° F.		Bacteriological Count: Less than 10,000		Bacteriological Count: From 10,000 to 50,000		Bacteriological Count: From 50,000 to 100,000		Bacteriological Count: From 100,000 to 1,000,000		Bacteriological Count: Over 1,000,000	
					No.	Percentage	No.	Percentage	No.	Percentage	No.	Percentage	No.	Percentage	No.	Percentage	No.	Percentage	No.	Percentage	No.	Percentage	No.	Percentage	No.	Percentage	No.	Percentage	No.	Percentage	No.	Percentage	No.	Percentage	No.	Percentage	No.	Percentage	No.	Percentage
January—																																								
Raw, cans	100	26	26	9	85	85	65	65	35	35	55	85			10	15	28	28	72	72	89	89	1	1	10	10					6	6	17	17	13	13	57	57	[illegible]	27
" bottles	100	23	23	1	98	98	73	73	27	27	45	62	25	34	3	4	95	95	5	5	82	82	13	13	5	5					9	9	25	25	9	9	41	41	16	[illegible]
Past., cans	100	2	2	6	35	35	90	90	10	10	56	62	21	23	13	15	100	100			92	92	8	8							71	71	13	13	[illegible]	[illegible]	10	10	1	1
" bottles	100	14	6	13	31	31	84	84	16	16	39	47	23	27	22	26	100	100			90	90					4	4	6	6	49	49	28	28	19	19	1	1		
Heated, bottles	100	14	7	13	24	24	48	48	52	52	30	62	12	25	6	13	97	97	3	3	77	77	21	21	2	2					16	16	29	29	27	27	23	[illegible]	[illegible]	[illegible]
April—																																								
Raw, cans	100	42	56	2	96	96	94	94	6	6	63	67	8	9	23	24	64	64	36	36	78	78	11	11	11	11					7	7	16	16	17	17	[illegible]	[illegible]	[illegible]	[illegible]
" bottles	100	20	22	3	88	88	100	100			78	78	18	18	4	4	100	100			79	79	17	17	4	4					7	7	17	17	16	16	49	49	11	11
Past., cans	100	5	5	6	39	39	67	67	33	33	23	34	36	54	8	12	85	85	15	15	41	41	50	50	9	9					55	55	33	33	4	4	7	7	1	1
" bottles	100	11	18	13	45	45	94	94	6	6	38	40	38	40	18	20	94	94	6	6	62	62	24	24	14	14					49	49	17	17	16	16	1	1	[illegible]	[illegible]
Heated, bottles	50	9	9	8	22	44	31	62	19	38	8	26	14	45	9	29	50	100			26	52	7	14	11	22	6	12			5	10	16	32	8	16	20	40	1	[illegible]
July																																								
Raw, cans	100	30	36		100	100	82	82	18	18	70	85			12	15	91	91	9	9	26	26	45	45	17	17	8	8	4	4	7	7	3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
" bottles	100	18	21		100	100	88	88	12	12	88	100					100	100			56	56	20	20	21	21	3	3					15	15	8	8	[illegible]	[illegible]	[illegible]	23
Past., cans	100	6	4	19	17	17	21	21	79	79	17	81	4	19			99	99	1	1	23	23	28	28	39	39	5	5	5	5	12	12	26	26	12	12	[illegible]	[illegible]	[illegible]	
" bottles	100	18	4	14	20	20	50	50	50	50	7	14	27	54	16	32	100	100			37	37	19	19	30	30	14	14			14	14	35	35	20	20	31	31		
Heated, bottles	50	4	1	12	5	10	19	38	31	62			19	100			50	100			6	12	25	50	16	32	3	6			9	18	25	50	7	14	[illegible]	[illegible]		
October																																								
Raw, cans	100	28	35	3	97	97	91	91	9	9	65	71	17	19	9	9	99	99	1	1	32	32	57	57	11	11					1	1	11	11	12	12	43	[illegible]	33	[illegible]
" bottles	100	[illegible]	27	2	91	91	97	97	3	3	85	88	6	6	6	6	100	100			77	77	13	13	10	10					6	6	10	10	8	8	37	[illegible]	[illegible]	[illegible]
Past., cans	100	6	4	12	22	22	28	28	72	72	5	18	22	78	1	4	96	96	4	4	87	87	5	5	8	8					65	65	18	18	11	11	[illegible]	[illegible]	3	[illegible]
" bottles	100	15	[illegible]	14	11	11	33	33	67	67	8	24	22	67	3	9	90	90	10	10	62	62	21	21	17	17					25	25	30	30	12	12	[illegible]	[illegible]	2	2
Heated, bottles	50	5	1	9	2	4	27	54	23	46	4	15	23	85			50	100			33	66	12	24	5	10					10	20	18	36	8	16	14	28		
Heated, cans, for 1911	100	7	6	17	37	37	55	55	45	45	31	56	19	35	5	9	90	90	10	10	51	51	7	7	41	41	1	1			23	23	30	30	14	14	32	[illegible]	1	1

On January 17 the lowest level reached was 16.0; January 18, 16.0; January 19, 15.6; January 20, 16.1; January 21, 13.9. The amount of water pumped into the reservoir during the eventful week is shown by meter readings as follows:

January 14,	243,600 gallons	January 18,	264,975 gallons
" 15,	229,600 "	" 19,	280,950 "
" 16,	804,600 "	" 20,	212,150 "
" 17,	251,550 "	" 21,	408,225 "

FIG. 6.—South wall of reservoir. † Shows location of privy just dismantled.

The gauge readings show that after the reservoir level had been lowered to 6.6 feet on the morning of January 16 no more water was drawn from the reservoir until after 9 A.M., January 17. Since there is abundant evidence that many cases of enteritis occurred in the night of January 16–17 contamination of the reservoir water by seepage may be dismissed as the first or sole cause of the outbreak. Whether or not it was a contributory or secondary factor must remain an open question. Some seepage may have taken place when the reservoir level was lowered to 6.6 feet on January 16, and this may have reinforced the contamination from other sources, but certainty on this point is plainly impossible. Examination of the reservoir when it was emptied for inspection on February

22 showed no extensive seepage, although some bacterial contamination on the north side was clearly apparent. On the whole it is our opinion that the reservoir, although dangerously placed and insufficiently protected, played a very minor part, if any at all, in the outbreak.

3. *The pumping pit and filter well.*—In the report on the enlargement of the Rockford water supply already referred to the

FIG. 7.—West end of reservoir on the right.

statement occurs: "We are informed that the bottom and sides of the suction well at the station freely admit the ground water whenever its level is higher than that of the water in the suction well." On further inquiry we found that it was generally admitted that both the suction well or pumping pit and the old filter well were pervious, and might under certain conditions permit entrance of the ground water. The use of these porous receptacles is plainly dangerous. The ground water in the neighborhood of the pumping station is highly contaminated. In addition to the numerous privies in use in the neighborhood, the whole sewerage system in this part of the city contributes to the pollution of the ground water. Sewers enter Rock River in the half-mile above the pump-

ing station at eight different points. The large Peach Street sewer empties into the river just below the pumping station (Fig. 1). Near the lower end of the city the river is dammed for power purposes, forming a pool about 10 feet in depth just above the dam. The crest of the dam has recently been raised about a foot, making the river level this year higher than before. At the waterworks the level of the river water is above the bottom of the pumping

FIG. 8.—North side of reservoir, showing privy within five feet of wall.

pit and filter well a large part of the time, and sometimes above the level of the reservoir bottom. The distance from the filter well to the river is only about 40 feet. All these facts indicated a critical condition.

The level in the suction well usually ranges, according to the waterworks officials, from 10 to 12 feet, but is not uncommonly reduced to eight feet. In the event of a heavy draft because of a fire, the pumps are speeded up and the level of the water lowered in a very short time. Since the level and the degree of contamination of the ground water must undoubtedly vary, the particular level at which contamination would take place could in no case be predicted. That seepage of ground water into the suction well

might take place was shown first by analyses, second by emptying the pit. Repeated bacterial examination of the water in the suction well when the level was 10–12 feet showed no trace of pollution, the plates being sterile or containing only three or four colonies. When, however, the water was lowered to $8\frac{1}{2}$ feet, a level said to be not infrequently brought about by slight changes in the rates at which the pumps are operated, the number of bacteria in the surface water increased to about 50 (37° C.). Samples taken four feet below the surface showed 1,500 colonies per c.c. The porosity of the pits was manifest when they were later emptied. On first stopping the pumps the pit level fell about a foot overnight, and when all the water was pumped out *about six feet "of refined sewage" seeped in before morning.* City officials no longer entertained any doubt as to the danger from this source. As one official expressed it: "We have been sitting on a keg of powder with a sputtering pipe in our mouths. That the explosion was not greater and more harmful than it was passes comprehension. We have had an unexcelled water source and then handled it as they did several decades ago—put our water storage in the middle of a cesspool and trusted to chance that it would not be contaminated."

No record is kept of the level of water in the suction pit, and the actual happenings in this respect on January 15 and 16 are not known.

It is plainly impossible to apportion exact responsibility for the epidemic between well No. 2, the reservoir, and the suction well. It may be pointed out that besides fluctuation in water level in the different receptacles for the deep well water, two other variable factors are concerned, namely, the level of the ground water and the degree of contamination of the ground water. Neither of these latter factors can be predicted or controlled with any degree of certainty. A given water level in any of the pervious underground receptacles of the water may at one time be sufficient to keep out ground water altogether, while at another it may allow seepage to enter. It is not possible, therefore, to assume that the condition of water collected at one time necessarily corresponds precisely with the condition of the water at an earlier period, even if the level within the receptacle were known to be the same. Unknown

and uncontrollable changes in the ground water are just as likely to bring about contamination under suitable conditions as changes in the level of the container.

The facts as set forth might be thought to indicate that before the present extensive outbreak occasional pollution had occurred from one or more of the sources previously considered, but it is plainly impossible to obtain proof of this view. In the spring of 1910 well No. 2 was fitted with a Norris nozzle and the engineer's report already cited states that "Owing to the obstruction in the casing of well No. 2, the Norris nozzle is said to have stuck at about 140 feet below the surface and attempts to lower it beyond this depth failed." It is uncertain whether there is any causal connection between this occurrence and the increased prevalence of typhoid fever since the spring of 1910. Water samples obtained directly from well No. 2, without admixture with the water of the pit, have given some evidence of contamination, but it has not been possible to obtain samples under entirely satisfactory conditions. The question needs further examination by local authorities before the well is again used.

The distribution of the enteritis cases throughout the school population is sufficiently peculiar to call for explanation (Fig. 2). The distribution of typhoid fever cases follows the enteritis distribution very closely (Fig. 3). It will be noted first that the whole northern section of the city is less severely affected than the southern and western sections; second that the region in which the furniture factory fire occurred on January 16 is among the slightly affected districts. The cause for this uneven distribution of the contaminated water is probably the relatively short time during which the extreme pollution continued, coupled with the greater demand for water made throughout the daytime by the factory and business districts. As already stated, the demand for water by the factories is greatest during the forenoon and early afternoon. On the morning of January 16 the increased demand for water was faced by an empty reservoir. The emergency was met by speeding up the pumps, this in turn drawing contaminated ground water into the suction well. This polluted water went where the draft was at the time greatest, namely, into the southern and western

districts, while the northern residential district, largely on high ground where the water pressure is at best low, received relatively little of the temporarily contaminated water. The contaminated water from well No. 2 also entered the system about noon at a time when the water demand from the southern section of the city was still high. On the following days whatever contamination might exist in the reservoir was distributed to the same section of the city.

The distribution of cases, somewhat unusual for a water-borne epidemic, is thus seen to depend upon (*a*) the temporary nature of the contamination of the water, and (*b*) the fact that at the time the contamination occurred the draft of water in certain districts was much heavier than in others. Those districts where the water demand was large throughout the daytime on January 16 were the ones that had a relatively large share of enteritis and typhoid fever cases.

The obvious recommendations were made. The use of well No. 2 was discontinued. Hypochlorite of lime was added to the reservoir and the reservoir used as a pumping pit while the suction pit at the pumping station and the old filter well were being made impervious to ground water. The usual precautions against infection of milk supplies were taken, and special measures for preventing later fly infection and contact infection were carried out by the health commissioner, Dr. W. E. Park. Abolition of privies and shallow wells was urged on the ground of possible future infection.

SUMMARY.

The Rockford outbreaks of enteritis (10,000 cases) in January, and of typhoid fever (199 cases) in February, 1912, were due to an unusual contamination of the public water supply occurring on January 16, 1912. The city supply was derived at the time of the outbreak entirely from deep wells. The deep well water, as it is drawn to the surface, is of unimpeachable quality, but the nature of the receptacles in which it is placed prior to distribution exposes it to pollution. These receptacles are porous pits located in a region where the ground water is highly charged with sewage. It

has been proved that the polluted ground water is able to enter these pits under conditions similar to those known to have obtained on January 16, a date when the demand upon the water system was unusually great, owing to a large factory fire. On the same date (January 16) a long-unused well was drawn upon as a source of supply. We have shown that the construction of this well and its mode of connection with the pumping station expose the water to contamination before it enters the general system. Under certain conditions of the ground water level this contamination, owing to the proximity of a large sewer, may be very gross. Since inspection and analysis have shown that both these sources of contamination exist and were undoubtedly operative under conditions prevailing on January 16, it is impossible to assign to either factor the sole or even the major responsibility for the outbreak. Possibly both were reinforced by a third factor, a slightly leaky reservoir drawn upon after the outbreak was under way, but this is uncertain.

We are under special obligation to his Honor, W. W. Bennett, mayor of Rockford, and Dr. Wm. E. Park, commissioner of health, for much assistance rendered us in the course of our investigation.

A STUDY OF DIPHTHERIA BACILLI WITH SPECIAL REFERENCE TO COMPLEMENT-FIXATION REACTIONS.*

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Because of the important relation of diphtheria to public health and the ease with which cultures usually may be made from the infected parts, the bacteriological diagnosis of diphtheria soon became an important adjunct in the diagnosis and management of this infection. It was soon discovered, however, that the bacteriology of diphtheria was not a simple matter, but that the bacillus might present a most varied morphology. Quickly a "group" of organisms was isolated, which centered about a chief type but differed in certain details.

The study of this group and especially the identity of the Hofmann bacillus and its relation to the diphtheria bacillus has engaged the attention of bacteriologists ever since it was discovered in 1886. Whether or not this pseudo-type could be made virulent or change its morphology to resemble the typical diphtheria bacillus, and vice versa, whether a true diphtheria bacillus could be made to resemble the pseudo-type, were problems which called forth extensive research with varied results. That the question is one of great importance is readily understood. This study bears specifically upon this question.

Among the first experiments carried out by Bordet and Gengou in their studies upon complement-fixation was the use of a bacterial antigen (plague) and its immune serum. It was found that complement was fixed in the presence of antigen and immune body but remained free if either of these were absent. A specific relation was supposed to exist between antigen and its immune body, and accordingly Wassermann, in evolving his practical sero-diagnosis of syphilis, used tissues rich in *Treponema pallidum*. Subsequently it was found that normal organs would suffice as antigens and

* Received for publication April 24, 1912.

that the lipoids are the active agents in such antigens, being brought into relationship with the immune body of the syphilitic serum through the agency of the globulins (Adami).

The reaction has been applied to the diagnosis of diseases other than syphilis by the substitution of specific antigens, notably in glanders and gonorrhea. The reaction of complement-fixation depends chiefly upon the fact that neither antigen nor immune body alone can fix complement, and that this fixation occurs only when both are present in certain proportions. Consequently if the antigen is known the immune body may be found, or conversely, if the immune body is known the antigen may be found.

With others we have been able to show that to a certain extent streptococci may be differentiated by complement-fixation reactions with homologous antigens.[1] The question then occurred to us whether or not it is possible to differentiate between the various types of diphtheria bacilli and in particular between the true diphtheria bacillus and the Hofmann's bacillus, by complement-fixation reactions with homologous antigens and immune sera. If, for instance, the serum of an animal immunized with a true diphtheria bacillus did not fix complement with an antigen of Hofmann's bacillus but did so with its own antigen, it would tend to show that the two organisms were different species. On the other hand, if each immune serum acted in the same manner with the antigens it would tend to show that while these organisms differ morphologically and biologically in some particulars yet essentially they are the same. The object of this study was as follows:

> "To determine the possibility of differentiating the various members of the diphtheria group of organisms by complement-fixation reactions, and particularly to determine the relation of Hofmann's bacillus, also termed the pseudo-diphtheria bacillus, to the true diphtheria organism."

Eight cultures, representing different types of bacilli according to Wesbrook's classification, were selected from different sources. These included one culture fulfilling the morphological, biological, and pathogenic characteristics of Hofmann's bacillus. The types were recorded after the bacilli were secured in pure culture and subcultured several times on serum media. Smears of these cul-

[1] *Arch. Inter. Med.*, 1912, 9, p. 220.

tures were made after incubating for 24 hours at 35° C. and stained with dilute carbol-fuchsin. The following table gives the source, type, sugar reactions, and results of virulence tests:

TABLE 1.

Number of Culture	Source of Culture	Type of Bacillus	Sugar Tests							Inoculation Tests			Result
			Saccharose	Glucose	Dextrin	Mannite	Levulose	Lactose	Inulin	Hours Grown in Broth	Weight of Pig in Grams	Dose in c.c.	
1.....	Clinical diphtheria	B and C	–	+	+	–	?	+	–	72	260	1.30	Died
2.....	Healthy penis	C_1 –	–	–	–	–	–	?	–	72	282	1.4	Negative
3.....	Park's bac. No. 8	Granular	–	+	+	–	?	+	–	72	274	1.3	Died
4.....	Throat conval. diphtheria	C	–	+	+	–	–	–	–	72	290	1.5	Negative
5.....	Otitis media	C_2	+	–	–	–	–	?	–	72	300	1.5	Negative
6.....	Nasal diphtheria	D_2	–	+	–	–	+	+	–	72	284	1.4	Died
7.....	Throat carrier case	C_2	–	–	–	–	?	+	–	72	260	1 3	Negative
8...	Normal nose	Hofmann's bacillus	–	–	–	–	–	–	–	72	275	2.5	Negative

For media Hiss's serum water with 1 per cent of the various sugars was used for the acid-production tests. Results were recorded after cultures had been grown for five days at 35° C.

Virulence tests were carried out with standard-weight pigs and cultures for inoculation were grown in 1 per cent slightly alkaline glucose broth for 72 hours at 35° C. with tubes slanted. Injections were made in the median line and animals observed for five days after injection for evidences of edema about the site of injection and symptoms of toxemia. Virulent cultures were retested with toxin-antitoxin mixture.

Immune sera.—These were prepared by immunizing a series of rabbits with eight to 10 weekly intravenous injections of emulsions of bacilli grown on blood serum media. The first injection of each culture was of organisms heated at 52° C. for one hour -subsequent injections were living cultures in increasing doses. A number of rabbits succumbed before the final sera were secured. No preservatives were added. Sera were kept in the ice-chest and inactivated before use.

Antigens.—These were prepared of the eight cultures as follows:

1. Grown on serum media for 24 hours and then examined by smear for purity of growth.

2. Washed off with sterile 0.85 per cent NaCl solution and strained if particles of media broke away.

3. Emulsions thoroughly centrifuged; supernatant fluid poured off and organisms in the bottom of the centrifuge tubes dried in a cold place over calcium chloride and sulfuric acid. After drying, the tubes were paraffined and placed in ice-box until all sera were ready.

4. The dried bacilli were then ground with powdered glass and shaken for 48 hours in 0.85 per cent NaCl solution containing 5 per cent phenol, using 0.5 gm. of bacilli in 10 c.c. of the solution.

5. Bacteria were then thrown down in a high-power electric centrifuge and the supernatant fluid stored in dark glass bottles in ice-chest.

Complement.—Pooled serum of two or more guinea-pigs titrated for exact unit with two units of antisheep hemolysin and 1 c.c. of 5 per cent suspension of washed sheep corpuscles.

Hemolysin.—Antisheep—diluted so that 0.001 c.c. equaled two units.

Corpuscle suspension.—Washed sheep's corpuscles made up in a 5 per cent suspension. One cubic centimeter used in each test.

Antigen, immune serum, and complement were mixed and the total volume brought up to 5 c.c. with the addition of 0.85 per cent NaCl solution. After tests and controls had been incubated for one hour at 37° C. hemolysin and corpuscles were added. Reincubated for two hours and then placed in refrigerator for 20 hours, when results were noted.

Antigen and immune sera are designated according to the corresponding number of culture. Thus antigen and immune serum No. 1 were made from culture No. 1 and so on.

Antigenic and anti-complementary values of the eight antigens were then determined as shown in Table 2.

The antigen proved fairly satisfactory and 0.08 c.c. was determined as the antigenic unit, although with sera 4 and 7 the degree of complement-fixation was slight but definite.

We were now ready for the most important part of the study, namely, to determine if the antigens would fix complement equally well with all of the different immune sera, or, conversely, if the

TABLE 2.

FIXING AND ANTI-COMPLEMENTARY POWER OF ANTIGENS.

Normal Serum c.c.	Amount of Antigen c.c.	Antigen No. 1	Antigen No. 2	Antigen No. 3	Antigen No. 4	Antigen No. 5	Antigen No. 6	Antigen No. 7	Antigen No. 8
0.2	0.04	C.H.*	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.2	0.06	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.2	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.2	0.1	C.H.	C.H.	C.H.	C.H.	C.H.	S.I.H.	S.I.H.	S.I.H.
0.2	0.15	C.H.	C.H.	C.H.	C.H.	S.I.H.	M.I.H.	S.I.H.	M.I.H.
0.2	0.18	S.I.H.	S.I.H.	C.H.	C.H.	S.I.H.	M.I.H.	M.I.H.	I.H.
0.2	0.2	M.I.H.	M.I.H.	S.I.H.	S.I.H.	M.I.H.	I.H.	I.H.	I.H.
0.2	0.3	I.H.	M.I.H.	M.I.H.	M.I.H.	I.H.	I.H.	I.H.	I.H.
Immune Serum c.c.									
0.2	0.04	S.I.H.	M.I.H.	S.I.H.	C.H.	M.I.H.	M.I.H.	C.H.	I.H.
0.2	0.06	S.I.H.	I.H.	S.I.H.	S.I.H.	I.H.	I.H.	C.H.	I.H.
0.2	0.08	M.I.H.	I.H.	M.I.H.	S.I.H.	I.H.	I.H.	S.I.H.	I.H.
0.2	0.1	I.H.	I.H.	I.H.	M.I.H.	I.H.	I.H.	S.I.H.	I.H.
0.2	0.15	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.	M.I.H.	I.H.
0.2	0.18	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.	M.I.H.	I.H.
0.2	0.2	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.
0.2	0.3	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.

* C.H. = complete hemolysis; S.I.H. = slight inhibition of hemolysis; M.I.H. = marked inhibition of hemolysis; I.H. = complete inhibition of hemolysis.

sera with the different antigens would fix complement. The following tables give the results after trying each serum with all of the antigens:

TABLE 3.

IMMUNE SERUM NO. 1.

Immune Serum c.c.	Amount of Antigen c.c.	Antigen No. 1	Antigen No. 2	Antigen No. 3	Antigen No. 4	Antigen No. 5	Antigen No. 6	Antigen No. 7	Antigen No. 8
0.04	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.06	0.08	S.I.H.	S.I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.08	0.08	M.I.H.	M.I.H.	S.I.H.	C.H.	S.I.H.	C.H.	C.H.	S.I.H.
0.1	0.08	I.H.	I.H.	M.I.H.	S.I.H.	S.I.H.	S.I.H.	S.I.H.	M.I.H.
0.15	0.08	I.H.	I.H.	I.H.	S.I.H.	M.I.H.	S.I.H.	S.I.H.	I.H.
0.18	0.08	I.H.	I.H.	I.H.	M.I.H.	I.H.	M.I.H.	M.I.H.	I.H.
0.2	0.08	I.H.	I.H.	I.H.	M.I.H.	I.H.	I.H.	M.I.H.	I.H.

TABLE 4.

IMMUNE SERUM NO. 2.

Immune Serum c.c.	Amount of Antigen c.c.	Antigen No. 1	Antigen No. 2	Antigen No. 3	Antigen No. 4	Antigen No. 5	Antigen No. 6	Antigen No. 7	Antigen No. 8
0.04	0.08	C.H.	M.I.H.	C.H.	C.H.	M.I.H.	C.H.	C.H.	I.H.
0.06	0.08	S.I.H.	M.I.H.	C.H.	C.H.	M.I.H.	C.H.	C.H.	I.H.
0.08	0.08	M.I.H.	I.H.	S.I.H.	C.H.	I.H.	C.H.	C.H.	I.H.
0.1	0.08	I.H.	I.H.	M.I.H.	S.I.H.	I.H.	C.H.	S.I.H.	I.H.
0.15	0.08	I.H.	I.H.	I.H.	M.I.H.	I.H.	M.I.H.	M.I.H.	I.H.
0.18	0.08	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.
0.2	0.08	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.

TABLE 5.

Immune Serum No. 3.

Immune Serum c.c.	Amount of Antigen c.c.	Antigen No. 1	Antigen No. 2	Antigen No. 3	Antigen No. 4	Antigen No. 5	Antigen No. 6	Antigen No. 7	Antigen No. 8
0.04	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.06	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.08	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.1	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	S.I.H.	C.H.
0.15	0.08	C.H.	C.H.	C.H.	C.H.	S.I.H.	C.H.	M.I.H.	C.H.
0.18	0.08	C.H.	S.I.H.	M.I.H.	S.I.H.	M.I.H.	C.H.	M.I.H.	S.I.H.
0.2	0.08	S.I.H.	M.I.H.	I.H.	M.I.H.	I.H.	S.I.H.	I.H.	M.I.H.

TABLE 6.

Immune Serum No. 4.

Immune Serum c.c.	Amount of Antigen c.c.	Antigen No. 1	Antigen No. 2	Antigen No. 3	Antigen No. 4	Antigen No. 5	Antigen No. 6	Antigen No. 7	Antigen No. 8
0.04	0.08	C.H.	C.H.	C.H.	C.H.	I.H.	M.I.H.	I.H.	....
0.06	0.08	C.H.	C.H.	C.H.	C.H.	I.H.	I.H.	I.H.	....
0.08	0.08	C.H.	C.H.	C.H.	S.I.H.	I.H.	I.H.	....	....
0.1	0.08	C.H.	S.I.H.	C.H.	M.I.H.	I.H.	I.H.	....	....
0.15	0.08	S.I.H.	M.I.H.	S.I.H.	I.H.	I.H.	I.H.	....	....
0.18	0.08	M.I.H.	I.H.	M.I.H.	I.H.	I.H.	I.H.	....	....
0.2	0.08	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.	....	....

TABLE 7.

Immune Serum No. 5.

Immune Serum c.c.	Amount of Antigen c.c.	Antigen No. 1	Antigen No. 2	Antigen No. 3	Antigen No. 4	Antigen No. 5	Antigen No. 6	Antigen No. 7	Antigen No. 8
0.04	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.06	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.08	0.08	C.H.	C.H.	C.H.	C.H.	S.I.H.	C.H.	C.H.	C.H.
0.1	0.08	C.H.	C.H.	C.H.	C.H.	M.I.H.	C.H.	C.H.	C.H.
0.15	0.08	C.H.	C.H.	C.H.	C.H.	M.I.H.	S.I.H.	S.I.H.	C.H.
0.18	0.08	S.I.H.	C.H.	C.H.	M.I.H.	I.H.	M.I.H.	S.I.H.	C.H.
0.2	0.08	S.I.H.	S.I.H.	S.I.H.	I.H.	I.H.	I.H.	M.I.H.	S.I.H.

TABLE 8.

Immune Serum No. 6.

Immune Serum c.c.	Amount of Antigen c.c.	Antigen No. 1	Antigen No. 2	Antigen No. 3	Antigen No. 4	Antigen No. 5	Antigen No. 6	Antigen No. 7	Antigen No. 8
0.04	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	S.I.H.
0.06	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	M.I.H.
0.08	0.08	C.H.	C.H.	C.H.	C.H.	S.I.H.	C.H.	C.H.	I.H.
0.1	0.08	C.H.	C.H.	C.H.	C.H.	S.I.H.	S.I.H.	C.H.	I.H.
0.15	0.08	C.H.	C.H.	C.H.	C.H.	M.I.H.	S.I.H.	C.H.	I.H.
0.18	0.08	S.I.H.	C.H.	C.H.	S.I.H.	I.H.	M.I.H.	C.H.	I.H.
0.2	0.08	M.I.H.	S.I.H.	S.I.H.	M.I.H.	I.H.	I.H.	S.I.H.	I.H.

TABLE 9.

IMMUNE SERUM NO. 7.

Immune Serum c.c.	Amount of Antigen c.c.	Antigen No. 1	Antigen No. 2	Antigen No. 3	Antigen No. 4	Antigen No. 5	Antigen No. 6	Antigen No. 7	Antigen No. 8
0.04	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.06	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.08	0.08	C.H.	C.H.	C.H.	C.H.	S.I.H.	C.H.	C.H.	C.H.
0.1	0.08	C.H.	C.H.	C.H.	C.H.	S.I.H.	C.H.	C.H.	S.I.H.
0.15	0.08	C.H.	C.H.	C.H.	C.H.	M.I.H.	C.H.	C.H.	S.I.H.
0.18	0.08	C.H.	S.I.H.	C.H.	C.H.	M.I.H.	C.H.	C.H.	M.I.H.
0.2	0.08	C.H.	S.I.H.	S.I.H.	C.H.	I.H.	C.H.	S.I.H.	M.I.H.

TABLE 10.

IMMUNE SERUM NO. 8.

Immune Serum c.c.	Amount of Antigen c.c.	Antigen No. 1	Antigen No. 2	Antigen No. 3	Antigen No. 4	Antigen No. 5	Antigen No. 6	Antigen No. 7	Antigen No. 8
0.04	0.08	C.H.	C.H.	C.H.	C.H.	I.H.	C.H.	C.H.	I.H.
0.06	0.08	C.H.	C.H.	C.H.	C.H.	I.H.	C.H.	C.H.	I.H.
0.08	0.08	C.H.	C.H.	C.H.	C.H.	I.H.	C.H.	C.H.	.
0.1	0.08	C.H.	C.H.	C.H.	C.H.	. .	C.H.	C.H.	.
0.15	0.08	S.I.H.	C.H.	S.I.H.	C.H.	. . . .	S.I.H.	C.H.	
0.18	0.08	M.I.H.	S.I.H.	S.I.H.	S.I.H.	. . .	M.I.H.	S.I.H.	
0.2	0.08	M.I.H.	M.I.H.	M.I.H.	S.I.H.	. . .	M.I.H.	M.I.H.	

Following these experiments the sera from seven cases of diphtheria were tried with the eight different antigens. These sera were collected four days previous to setting up the tests; sera were inactivated by heating to 55° C. for 25 minutes.

CASE 1.

J. B.; age 6 years; tonsillar diphtheria due to diphtheria bacillus, type C. Patient had received 5,000 units of antitoxin four days ago:

Serum c.c.	Amount of Antigen c.c.	Antigen No. 1	Antigen No. 2	Antigen No. 3	Antigen No. 4	Antigen No. 5	Antigen No. 6	Antigen No. 7	Antigen No. 8
0.05	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.1	0.08	S.I.H.	C.H.	S.I.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.2	0.08	M.I.H.	C.H.	M.I.H.	C.H.	C.H.	C.H.	C.H.	C.H.

CASE 2.

H. R.; age 4 years and 9 months; developed tonsillar diphtheria three days ago; diphtheria bacillus, type C; received 5,000 units of antitoxin three days ago:

Serum c.c.	Amount of Antigen c.c.	Antigen No. 1	Antigen No. 2	Antigen No. 3	Antigen No. 4	Antigen No. 5	Antigen No. 6	Antigen No. 7	Antigen No. 8
0.05	0.08	C.H.	C.H.	S.I.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.1	0.08	S.I.H.	S.I.H.	M.I.H.	S.I.H.	C.H.	C.H.	S.I.H.	C.H.
0.2	0.08	M.I.H.	M.I.H.	I.H.	S.I.H.	C.H.	C.H.	S.I.H	C.H.

CASE 3.

D. R.; age 12 years; developed tonsillar diphtheria six days ago, type C. Received unknown quantity of antitoxin previous to admittance to hospital.

Immune Serum c.c.	Amount of Antigen c.c.	Antigen No. 1	Antigen No. 2	Antigen No. 3	Antigen No. 4	Antigen No. 5	Antigen No. 6	Antigen No. 7	Antigen No. 8
0.05	0.08	C.H.	C.H.	C.H.	S.I.H.	C.H.	C.H.	C.H.	C.H.
0.1	0.08	S.I.H.	S.I.H.	S.I.H.	S.I.H.	S.I.H.	C.H.	C.H.	C.H.
0.2	0.08	M.I.H.	M.I.H	M.I.H.	M.I.H.	M.I.H.	S.I.H.	S.I.H.	C.H.

CASE 4.

F. B.; age $4\frac{1}{2}$ years; developed tonsillar diphtheria five days ago, type C_2 bacillus. Received 4,000 units of antitoxin four days ago·

Serum c.c.	Amount of Antigen c.c.	Antigen No. 1	Antigen No. 2	Antigen No. 3	Antigen No. 4	Antigen No. 5	Antigen No. 6	Antigen No. 7	Antigen No. 8
0.05	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.1	0 08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	S.I.H.
0.2	0.08	C.H.	S.I.H.	C.H.	C.H.	S.I.H.	C.H.	C.H.	M.I.H.

CASE 5.

S. S.; age 14 years; developed tonsillar diphtheria seven days ago, type C bacillus. Received 10,000 units of antitoxin six days ago:

Serum c.c.	Amount of Antigen c.c.	Antigen No. 1	Antigen No. 2	Antigen No. 3	Antigen No. 4	Antigen No. 5	Antigen No. 6	Antigen No. 7	Antigen No. 8
0.05	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.1	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.2	0.08	C.H.	C.H.	S.I.H.	C.H.	S.I.H.	S.I.H.	C.H.	C.H.

CASE 6.

J. K.; age 12 years; developed tonsillar diphtheria 11 days ago, type A bacillus. Received 10,000 units of antitoxin eight days ago:

Serum c.c.	Amount of Antigen c.c.	Antigen No. 1	Antigen No. 2	Antigen No. 3	Antigen No. 4	Antigen No. 5	Antigen No. 6	Antigen No. 7	Antigen No. 8
0.05	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.1	0.08	C.H.	C.H.	C.H.	C.H.	S.I.H.	C.H.	C.H.	S.I.H.
0.2	0.08	C.H.	C.H.	C.H.	S.I.H.	M.I.H.	C.H.	C.H.	M.I.H.

CASE 7.

J. C.; age 12 years; developed tonsillar diphtheria 14 days ago, type C bacillus. Received 10,000 units of antitoxin 10 days ago:

Serum c.c.	Amount of Antigen c.c.	Antigen No. 1	Antigen No. 2	Antigen No. 3	Antigen No. 4	Antigen No. 5	Antigen No. 6	Antigen No. 7	Antigen No. 8
0.05	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.1	0.08	C.H.	S.I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.2	0.08	C.H.	M.I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.

RESULTS.

While a study of the tables shows in a few instances a tendency to specific reaction, yet as a whole the results indicate most conclusively that all the immune sera and antigens acted in quite a similar manner, and, as far as complement-fixation tests are capable of indicating specific relations between immune body and its antigen these reactions show that each of the different types of diphtheria bacilli, including Hofmann's bacillus, are capable of causing the production of similar immune bodies.

CONTROLS.

That there is developed in the serum of an animal immunized with diphtheria bacilli a body capable of fixing complement only with its homologous antigen is seen in the following two tables where different antigens were used with the various diphtheria immune sera with uniform failure of complement-fixation:

TABLE 11.

Antigen of alcoholic extract of fetal syphilitic liver; antigenic for syphilitic antibody in amount of 0.2 c.c. of 1:3 dilution; becomes anti-complementary in amounts of 0.9 c.c. (dil. 1:3).

Serum c.c.	Amount of Antigen c.c.	Serum 1	Serum 2	Serum 3	Serum 4	Serum 5	Serum 6	Serum 7	Serum 8
0.04	0.2	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.06	0.2	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.08	0.2	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.1	0.2	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	S.I.H.
0.15	0.2	C.H.	C.H.	C.H.	S.I.H.	S.I.H.	C.H.	C.H.	S.I.H.
0.18	0.2	C.H.	C.H.	C.H.	M.I.H.	M.I.H.	C.H.	C.H.	M.I.H.
0.2	0.2	S.I.H.	C.H.	S.I.H.	I.H.	I.H.	S.I.H.	C.H.	M.I.H.

TABLE 12.

Antigen of aceton insoluble lipoids; antigenic with syphilitic antibody in amounts of 0.1 c.c.; becomes anti-complementary in amounts of 0.45 c.c.

Serum c.c.	Amount of Antigen c.c.	Serum 1	Serum 2	Serum 3	Serum 4	Serum 5	Serum 6	Serum 7	Serum 8
0.04	0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.06	0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.08	0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.1	0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	S.I.H.
0.15	0.1	C.H.	C.H.	C.H.	C.H.	S.I.H.	C.H.	C.H.	M.I.H.
0.18	0.1	C.H.	C.H.	C.H.	C.H.	M.I.H.	S.I.H.	C.H.	I.H.
0.2	0.1	S.I.H.	C.H.	C.H.	S.I.H.	I.H.	M.I.H.	C.H.	I.H.

These results indicate that the diphtheria bacillus causes the production of a body capable of fixing complement only with its

homologous antigen. The results with the two antigens of syphilitic liver and aceton insoluble lipoids were quite similar and with the exception of serum No. 8 showed uniform lack of power of fixing complement with the diphtheria antibody present in the immune sera. However, as already shown, the complement-fixing body of the diphtheria bacillus is the same for all types, including the Hofmann's bacillus, which organism is believed by some to be a separate entity.

In order to test still further the relation between diphtheria antigens and their immune sera, typhoid and streptococcus sera were tested with the eight different diphtheria antigens in order to ascertain whether the immune bodies present in these sera were capable of fixing complement with the diphtheria antigens:

TABLE 13.

Typhoid immune serum; agglutinates in dilution of 1:1,500 (macroscopical test); is only slightly antigenic with its own antigen. Tested with the eight diphtheria antigens as follows:

Serum c.c.	Antigen c.c.	Antigen 1	Antigen 2	Antigen 3	Antigen 4	Antigen 5	Antigen 6	Antigen 7	Antigen 8
0.04	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.06	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.08	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.1	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.15	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.18	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	S.I.H.	C.H.	C.H.
0.2	0.08	C.H.	S.I.H.	C.H.	S.I.H.	C.H.	M.I.H.	C.H.	C.H.

TABLE 14.

Anti-streptococcus serum; produced by a large number of injections of 16 different strains of scarlet fever streptococci into a horse; the serum in amounts of 0.08 c.c. fixes complement with 0.09 c.c. of antigen of scarlet fever streptococci. Tested with the eight diphtheria antigens as follows:

Serum c.c.	Amount of Antigen c.c.	Antigen 1	Antigen 2	Antigen 3	Antigen 4	Antigen 5	Antigen 6	Antigen 7	Antigen 8
0.04	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.06	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.08	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.1	0.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.15	0.08	S.I.H.	S.I.H.	S.I.H.	S.I.H.	S.I.H.	S.I.H.	S.I.H.	S.I.H.
0.18	0.08	S.I.H.	S.I.H.	M.I.H.	M.I.H.	M.I.H.	M.I.H.	M.I.H.	M.I.H.
0.2	0.08	M.I.H.	M.I.H.	I.H.	M.I.H.	I.H.	M.I.H.	I.H.	I.H.

An examination of these results shows that as a rule complement is not fixed by typhoid and streptococcus immune bodies and diphtheria antigens and that therefore the relation between diphtheria antigens and immune body is more or less specific.

RELATION BETWEEN DEGREE OF COMPLEMENT-FIXATION AND ANTITOXIN CONTENT OF DIPHTHERIA IMMUNE SERA.

The results of complement-fixation tests with sera secured from three different antitoxin laboratories and nine different antigens of diphtheria bacilli, including the same bacilli used in the production of these antitoxins, shows no relation between the occurrence or degree of complement-fixation and the antitoxic strength of the serum. These studies were then extended along similar lines, using other organisms and their immune sera. The results of this study are given in a separate communication. While the number of antitoxin units in the eight sera used in these experiments was not determined, yet the results with sera of known strengths demonstrated clearly the lack of any relation between complement-fixation and antitoxin content and indicated that in complement-fixation we are probably dealing with a body in the serum, in the nature of an amboceptor, not a true antibody in the sense of being prophylactic or curative.

HOFMANN'S BACILLUS (PSEUDO-DIPHTHERIA BACILLUS).

Most of the controversy in the bacteriology of diphtheria concerns this organism. A great amount of work has been done in an effort to establish its identity and its relation to the true diphtheria bacillus. By various ways, including passage through different animals, growth upon different culture media, acid production with different sugars, and straining reactions, attempts were made to change the morphological, biological, and pathogenic properties of this organism to make it resemble the true granular types of diphtheria bacilli. Conversely, attempts were made to convert true diphtheria bacilli into the pseudo-types. As a rule the sum total of the various work recorded indicates that Hofmann's bacillus possesses such characteristics as to permit its differentiation from the true type of diphtheria bacilli. The results of this complement-fixation work indicate that it belongs essentially to the diphtheria group and is not a separate entity in the sense of being totally unrelated. It would appear that this bacillus has been changed in its chemical reaction and structure, as a result of being hard pressed, and has gradually developed a state of effective resistance

or "fastness" to unfavorable surroundings. The new qualities acquired may thus be considered the result of "mutation" (Flexner) among the members of this group of organisms. As a result an organism has been evolved, "a mutant," capable of transmitting its new characteristics through an indefinite number of generations. The Hofmann bacillus is the best marked example of mutation among the diphtheria group, but as would be expected many other strains may be found with partially fixed characteristics, as, for instance, many of the strains of the D_2 type which closely resemble Hofmann's bacillus with the exception of being able to produce acid with one or more of the sugars. To these the unfortunate and hopeless term "pseudo-diphtheria" has been applied. It would be better to call them non-virulent diphtheria bacilli.

CONCLUSIONS.

1. Complement-fixation reactions with homologous antigens and immune sera of different types of diphtheria bacilli from a variety of clinical conditions, including a true Hofmann's bacillus, tend to show that these organisms are all related.

2. The true Hofmann's bacillus is probably an example of "mutation" and is able to transmit its new qualities from generation to generation.

3. There is no relation between the occurrence or degree of complement-fixation and antitoxic strength of diphtheria immune sera. It seems that complement is fixed by a "body" separate from the true immune body representing the antitoxic content of sera.

A CONTRIBUTION TO THE BACTERIOLOGY OF DIPHTHERIA.*

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Interest in the bacteriology of diphtheria has been directed to the study of the identity of Hofmann's bacillus and the group of "pseudo-diphtheria bacilli," and their relation to the true diphtheria bacillus; to study of methods and means whereby a virulent bacillus may be differentiated from the non-virulent in practical culture work; and to the study of the question of "carriers," from both a bacteriological and public health point of view.

Ever since the discovery of Hofmann's bacillus efforts have been made to establish its relation to the true diphtheria bacillus. Various culture media, differential stains, and passage through various animals have been tried in the endeavor to make it virulent or change its morphology. Similar efforts have been made to convert a typical diphtheria bacillus into the Hofmann type. The results reported have not been uniform, although present indications point to the fact that the Hofmann bacillus possesses such characteristics as to justify its recognition as a separate entity. In my paper on "A Study of Diphtheria Bacilli with Special Reference to Complement-Fixation Reactions,"[1] however, it has been shown by complement-fixation tests with homologous antigens and immune sera of different types of diphtheria bacilli from varied clinical sources, including a true Hofmann bacillus, that all of these bacilli are closely related and that the Hofmann bacillus belongs to the group of diphtheria bacilli, but that as a result of various influences the latter has been changed in its chemical reactions, structure, and virulence so as to give it constant and more or less new characteristic features which are transmitted through a number of generations. Between this decided example of "mutation" and the parent or true diphtheria bacilli are many

* Received for publication April 24, 1912.

[1] This Journal, p. 44.

bacilli partaking of some of the features of both. Hence the development of the large indefinite "group" vaguely termed "pseudo-diphtheria" bacilli.

The question of bacillus "carriers" is especially troublesome when one is investigating an epidemic or endeavoring to control diphtheria infection by extensive culture work. To this day the great number of practitioners do not understand why a patient who is apparently fully recovered may still be dangerous to others. In practice it is not possible to isolate every person found, as a result of extensive culture work, to be harboring diphtheria-like bacilli. The "carrier" of virulent diphtheria bacilli is probably immune because of the presence of antitoxin in his blood, or the bacilli may be regarded as examples of "mutation," in that while they have at one time caused disease in the host they have now become adapted to their environment and are harmless to him but not to others.

While it is possible to increase the virulence of an organism by repeated "passage" through the lower animals, it has not definitely been proved possible to give virulence to a known non-virulent organism; in diphtheria culture work we are constantly encountering these non-virulent bacilli. Hence any method enabling the bacteriologist to differentiate between virulent and non-virulent bacilli with a reasonable degree of accuracy and without resorting to expensive and prolonged animal inoculation and acid-production tests would be of great value in the proper management of diphtheria. Largely with this end in view a morphological classification was proposed by Wesbrook, Wilson, and McDaniel, wherein the various bacilli were regarded as dangerous, doubtful, or negligible according to their size and structure. According to this plan differentiation could be made within 24 hours after taking the culture. The value and limitations of the classification can be determined only after extensive comparative bacteriological and clinical studies with a large number of animal inoculation tests for virulence.

The object of this study is therefore as follows:

1. To investigate the relation of types of diphtheria bacilli to clinical diphtheria, with special attention to the value of a mor-

phological classification in bacteriological diagnosis and management of diphtheria.

2. To investigate animal inoculation tests, especially as concerns their value in the management of prolonged positive culture and "carrier" cases.

3. To investigate the value of acid-production tests in differentiating members of the diphtheria group of bacilli.

4. To give practical discussion of the bacteriological diagnosis and management of diphtheria.

TYPES OF BACILLI.

According to the morphological classification of Wesbrook, Wilson, and McDaniel, the various kinds of diphtheria bacilli may be divided into three main groups: granular, barred, and solid bacilli. Each of these groups is subdivided into types according to the size and shape of bacilli, the number and distribution of granules, number of bars, etc. In practical work the routine use of such a classification presents a few difficulties. Many cultures show bacilli of different groups side by side or different types of the same group, so that it is frequently difficult to classify. The differences between some of the types are often so slight as to require high magnification and great care in differentiating. Differences in the length of incubation of cultures and temperature of incubator will frequently alter the results. Thus after short incubation solid types predominate which later may become granular; an incubator temperature over 37° or 38° C. results in the growth of short forms difficult to recognize and classify. Cultures should be incubated for at least 18 hours at 35° C. Ordinary Loeffler's methylene blue is the best routine stain.

The predominating type of organism was recorded in cultures of 253 cases of diphtheria from early in the infection until two successive negative cultures were obtained. In the primary cultures the following types predominated:

A. Tonsillar Diphtheria		186 cases
1. Granular bacilli	Types AA, B, and C (mostly C)	83.8 per cent
2. Barred bacilli	Type C_1	.5 " "
3. Solid bacilli	Type A_2	2.6 " "
	Type C_2	9.7 " "
	Type D_2	3.2 " "

B. Tonsillar and Laryngeal Diphtheria			20 cases
1. Granular bacilli	Types A, B, and C (mostly C)	80.0	per cent
2. Solid bacilli	Type A_2	2.0	" "
	Type C_2	10.0	" "
	Type D_2	8.0	" "
C. Laryngeal Diphtheria			39 cases
1. Granular bacilli	Types A, B, and C (mostly C)	69.2	per cent
2. Solid bacilli	Type A_2	5.1	" "
	Type C_2	20.5	" "
	Type D_2	5.1	" "
D. Nasal Diphtheria			8 cases
1. Granular bacilli	Mostly C	50.0	per cent
2. Solid bacilli	Type C_2	25.0	" "
	Type D_2	25.0	" "

The granular types of bacilli predominate in the largest proportion of cases of clinical diphtheria whether the disease is located in the nose, pharynx, or larynx. These types are likely to prove virulent even in prolonged culture cases and contacts, and should always be considered dangerous and positive until proved otherwise, regardless of the clinical aspect or history of the case.

Our experience with the barred types occurring early in diphtheria is quite limited. They are distinctly rare about Philadelphia, and are more likely to be found in cultures taken late in the disease. We consider them similar to the granular types, although the barred types are frequently harmless non-virulent bacilli and may be found in 20 per cent of cultures from the healthy penis.

The solid types give the most concern. We have often encountered cultures of extensive exudates in severe clinical diphtheria which showed a few scattered solid bacilli scattered among cocci or diplococci. Secondary cultures are usually of granular types. To disregard the few solid bacilli in the primary culture would frequently throw the clinician off guard and be the cause of much mischief. Many primary cultures from severe cases may be entirely free of diphtheria bacilli. These results are frequently due to faulty methods in making the culture, for if one is satisfied with a light perfunctory swabbing over the exudate, only the organisms of secondary infection may be secured and not the diphtheria bacilli deep in the exudate and near the living tissues. On the other hand, "carrier" cases are likely to show the presence of a solid type of bacillus. Nevertheless, these bacilli, A_2, B_2, and

C_2, should be held until subsequent cultures are secured. If the patient is entirely well and runs a prolonged course of positive cultures the bacilli should be tested for virulence, to avoid a needlessly prolonged quarantine.

The short, solid D_2 type of diphtheria bacillus resembles Hofmann's bacillus and is especially troublesome in nose cultures. Occasionally it is virulent and is the only bacillus to be found in a case of clinical diphtheria. It is especially found in "carrier" cases and is therefore frequently encountered in cultures from suspected "contacts." In the great majority of cases if two consecutive cultures of a person free from clinical evidences of diphtheria shows the presence of this organism the results may be considered negligible. This type of diphtheria bacillus should not be confounded with Hofmann's bacillus. Both may prove non-virulent in massive doses to a guinea-pig, but if acid is produced with any of the sugars the culture is a true D_2 type—a non-virulent diphtheria bacillus. If acids are not produced with the sugars it is likely to be Hofmann's bacillus.

As a general rule, we believe that the morphological classification of Wesbrook, Wilson, and McDaniel is good. The recognition of types requires experience and frequent checking up of opinions by isolating and studying cultures. The method has certain drawbacks and requires much care, but in routine work it is of distinct value. If one could always be guided by bacteriological and clinical findings in the management of diphtheria even better results would be secured. But clinical ability and opinions differ a great deal among practitioners and few are capable of co-relating the clinical and bacteriological data.

CHANGEABILITY OF TYPES OF BACILLI.

Another interesting feature in the bacteriology of diphtheria is the change in types during the course of the disease. Solid types of bacilli are found more frequently at the end of the course than at the beginning. Cobbett claims that solid types are usually present from the beginning but in the early cultures are overshadowed by the granular bacilli and subsequently when these disappear the solid types become prominent.

In the 253 cases referred to above the culture records show the following results:

1. Granular types persisting throughout.........	55.4 per cent
2. Solid types persisting throughout.............	15.2 " "
3. Granular types replaced by solid types........	24.7 " "
4. Solid types replaced by granular types........	4.5 " "

It will be noted that in about 70 per cent of cultures the bacilli adhere to their group characteristics. We believe with Cobbett that the transformation of types is in most cases only apparent. A study of cultures carried through pigs and subcultured over a period of time shows that while the types of a group may change, as A into C, or a C_2 type become shorter and resemble D_2, yet the complete transformation of a granular bacillus into a solid, or vice versa, is decidedly uncommon. Not infrequently, however, a culture of D_2 may be found to show granular types after longer incubation, especially in cultures from clinical diphtheria. The replacement of solid by granular types often means reinfection of the patient, with the formation of a pseudo-membrane and the general symptoms of diphtheria.

ANIMAL INOCULATION TESTS.

Loeffler originally found that virulent diphtheria bacilli produce more or less characteristic changes when injected into a guinea-pig, prominent among which is the development of gelatinous edema at the site of injection. Since then abundant evidence has accumulated in support of his findings, and with a suitable technic the virulence of a given culture is readily determined. A question of greater importance, however, is whether an organism found non-virulent for the guinea-pig may regain its virulence when transferred to human tissues. In most cases it is comparatively simple to restore virulence to a culture by repeated "passage" through animals, hence the question regarding the delicacy of the pig-test for virulence. Are the tissues of the animal delicate enough to react to the influence of a slightly virulent culture? If a given culture were found to produce no disturbances in a pig and yet produce diphtheria in the human being, then the inoculation test

as a routine procedure is a mistake, and dangerous on account of its misleading information.

The practical benefits of such tests may be considerable because in this manner a quarantine may be lifted from a person or family suffering hardship by reason of prolonged isolation and detention. The attending physician may regard lengthy quarantine as a reflection upon his ability and a reversal of his opinion regarding the condition of his patient, and he may in this manner suffer in professional reputation. This, leading to antagonism between public officials and practitioners, is detrimental to public health, for without the hearty co-operation of the latter little real good can be accomplished.

It is understood, therefore, that while inoculation tests are often of much aid in the practical management of diphtheria they are, at least when conducted on a large scale, expensive, laborious, and time consuming. In an experimental way they are of much value in differentiating between true diphtheria bacilli and Hofmann's bacillus when used in conjunction with acid-production tests.

On the other hand, the differentiation between dangerous, doubtful, and harmless diphtheria bacilli according to their morphology is quick, comparatively easy, and inexpensive. Such a method has technical difficulties, as already dwelt upon, and is dangerous in the hands of the inexperienced. Nevertheless, experience has taught that such a method has distinct value, and when used together with judicious animal inoculation tests a highly satisfactory technic results.

Many observers have noted that diphtheria bacilli retain their virulence until they disappear, regardless of the length of their stay. Judging from our own results, we cannot agree entirely with this view. After a time, the minimum being not less than two weeks, a proportion of bacilli prove non-virulent when tested by animal inoculation. Whether or not they regain virulence when passed from throat to throat is the important question, difficult to answer because the disease may be spread in such a varied and confusing manner. We have no direct evidence, however, that this occurs. Most physicians depend upon two consecutive nega-

tive cultures before discharging a patient, but when one stops to consider the fallacies of such a method and the goodly proportion of those who will yield a positive culture if cultured the third time, showing thereby that they are not really free of the bacilli, it is a question whether or not a good pig-test is not to be preferred.

We believe that in some instances the prolonged number of positive cultures following diphtheria are due to the presence of harmless diphtheria-like bacilli of "carriers," such as are found in about 12 per cent of healthy throats and about 20 per cent of healthy noses, and having no connection with clinical diphtheria. They were present before the attack of diphtheria and are likely to remain for an indefinite time afterward. At first they are overgrown by the disease-producing organisms, but as these disappear the "mutants" become prominent again and yield a formidable line of positive cultures. A pig-test is certainly justifiable in cleaning up such a case.

If the animal inoculation test is to be accepted as an indication of the virulence or non-virulences of a given bacillus the technic must be satisfactory. Almost any procedure suffices when dealing with highly virulent bacilli, providing the bacilli are injected into the pig. But when dealing with bacilli of decreased virulence certain steps in technic become important. With a slightly virulent culture it is easily demonstrated that results vary according to differences in technic. The most important factor in inoculation work is the detection of slightly virulent cultures, because it has been abundantly proved that these may become fully virulent by "passage" through animals and probably also by transfer from throat to throat.

It should also be emphasized that in the same culture tube colonies of virulent and non-virulent bacilli may be growing side by side, and if a single colony is chosen for growing the culture for injection in conducting a pig-test the non-virulent may be picked out. To choose many colonies and test each separately requires many animals and becomes expensive. This difficulty may be obviated somewhat by inoculating a tube of broth with many different colonies. If sufficient time is permitted for growth the virulent bacilli will multiply to such an extent as to make their presence known.

The following 237 animal inoculation tests were conducted routinely in the Laboratory of the Philadelphia Hospital for Contagious Diseases with cultures from patients who had recovered from diphtheria and were running positive cultures. It also includes a smaller number of "carrier" cases, yielding positive cultures of diphtheria bacilli but presenting no clinical evidences of disease. From time to time a pig-test, not included here, was conducted with cultures of different types of bacilli from clinical cases, as controls over the technic. The results of these are considered in the conclusions bearing upon this subject.

TECHNIC.

a) *Types of bacilli.*—For the sake of brevity all the types found in the cultures are not recorded. In a given culture of granular bacilli types A, B, and C may be found, although if one predominates, the culture is recorded according to that type. The greater proportion of granular bacilli are of those three types and seem to be of equal virulence. We rarely find a true banded bacillus and have no record of a pig-test with bacilli of that type. Considerable difficulty frequently occurs in drawing sharp lines among the solid bacilli. If the temperature of the incubator goes up over night the bacilli are found short next morning and as a result a different type may be recorded. The A_2 type is usually easy of detection; to differentiate between B_2 and C_2 and between D_2 and E_2 is frequently most difficult. Most of our long solid types we have classified as C_2 and the short types, D_2. The cultures for the pig-test were recorded as follows:

A. Granular bacilli, A, B, C, and D—mostly C.
B. Solid bacilli:
    Long solid—A_2, B_2, and C_2—mostly C_2.
    Short solid—D_2 and E_2—mostly D_2.

b) *Animal inoculation test.*—

1. Isolation by "streak" method.

2. Examination of colonies; inoculation of a tube, containing 6 to 8 c.c. of neutral or slightly alkaline broth containing 1 per cent glucose, with several different colonies.

3. Broth culture slanted and grown for 72 hours at 35° C.

4. Examination of broth cultures for purity of growth. A healthy guinea-pig weighing between 250 and 300 gms. is injected in the median abdominal line with a dose of culture corresponding to 0.5 per cent of the body weight of the animal expressed in cubic centimeters. Thus a 260 gm. pig receives 1.3 c.c. of culture.

5. The animal is kept under observation for at least four days and examined for edema at the site of injection and for evidences of toxemia.

6. In case of death autopsy is performed and cultures made from site of injection and various internal organs, including the peritoneal cavity. The test is regarded as positive and a second pig injected with toxin-antitoxin mixture to determine more conclusively if death was due to diphtheria bacilli.

Certain points in technic require special mention. The reaction of the broth should be neutral or slightly alkaline because diphtheria bacilli produce acid rapidly, especially with glucose, and high acidity inhibits and finally kills the bacilli. Glucose aids in growing richer cultures. Inoculation of broth from solid serum media may require previous "education" by several daily subcultures before a good broth growth is secured. Animals over 300 gms. are too resistant to diphtheria to be safe for inoculation work. We feel reasonably certain that when the bacilli have once been "educated" to grow in broth sufficient toxins are elaborated in 48 hours to produce a positive result when injected in the dose recommended. For fear of missing a slightly virulent culture, however, we have extended the time by 24 hours. This extension does not make any material difference, because it takes at least a week to conduct the test under the best of circumstances. A toxic animal is regarded as a positive result, even though it does not succumb, until proved otherwise by toxin-antitoxin injection.

c) *Results.*—

A. Cultures from Throat			91
1. Granular types (mostly C)		60	
Positive	63.2 per cent		
Negative	36.6 " "		
2. Solid types (mostly C_2)		23	
Positive	26.0 " "		
Negative	74.0 " "		

3. Solid types (mostly D_2)		8	
Positive	12.5 " "		
Negative	87.5 " "		
B. Cultures from Nose			68
1. Granular types (mostly C)		25	
Positive	56.0 per cent		
Negative	44.0 " "		
2. Solid types (mostly C_2)		23	
Positive	26.0 per cent		
Negative	74.0 " "		
3. Solid types (mostly D_2)		20	
Positive	15.0 per cent		
Negative	85.0 " "		
C. Cultures from Ears			78
1. Granular types (mostly C)		27	
Positive	47.0 per cent		
Negative	53.0 " "		
2. Solid types (mostly C_2)		24	
Positive	8.0 per cent		
Negative	92.0 " "		
3. Solid types (mostly D_2)		27	
Positive	6.0 per cent		
Negative	94.0 " "		

d) *Conclusions.*—

1. The granular types of bacilli gave the highest percentage of positive tests. In clinical diphtheria they are almost invariably virulent for guinea-pigs. No matter how long they persist in the throat after recovery they should be regarded as dangerous until proved otherwise. Of all types they are most likely to retain their virulence.

2. The value of animal inoculation tests is especially appreciable when dealing with solid types of bacilli. The longer types are more likely to retain virulence than the shorter varieties. Both, however, may be descendants of the "carrier" bacillus rather than of the bacillus producing the clinical evidences of the disease.

3. The short solid types of bacilli are especially common in the nose in the absence of clinical diphtheria. In a small percentage of cases these bacilli are truly pathogenic but the majority of cultures are found without virulence when tested by animal inoculation.

4. The long solid types of bacilli, so frequently found in otitis media, are in the majority of cases non-virulent. We have tested

over 30 cultures of these bacilli isolated from cases of suppurative otitis media (not included in this series) with uniformly negative results.

5. Animal inoculation tests have a special field of usefulness in testing the solid types for virulence when they persist in the tissues over a long period of time or when found in contact and "carrier" cases.

ACID-PRODUCTION TESTS.

Differentiation among members of the diphtheria group of bacilli has been studied by means of acid-production tests, the bacilli differing in their power to break up certain sugars with the formation of acids. Such tests have been advocated as a means of studying and classifying the Hofmann bacillus and *B. xerosis*.

We have tested 62 cultures of various types of bacilli from clinical cases of diphtheria of the eye, ear, nose, and throat, and also from "carrier" cases, according to the following technic:

1. Hiss's serum-water media were used, colored with azolitmin for indicator and containing, respectively, 1 per cent of the following sugars: glucose, saccharose, dextrin, lactose, maltose, galactose, and mannite. The purest products only should be used. The lactose media frequently become acid and give trouble. Non-inoculated controls were always incubated along with the cultures.

2. Old cultures were "educated" to grow in the new media by two or more daily subinoculations.

3. Results were recorded after cultures had been incubated for five days at 35° C. The presence of acid was shown by the change in color—the blue being turned to pink or red.

The titration of total acidity in glucose broth cultures with sodium hydrate, with phenolphthalein as indicator, yields interesting and more important results, especially when studying the Hofmann bacillus.

The results of the work may be expressed as follows:

1. Virulent diphtheria bacilli produced acid most frequently with dextrose, next in frequency with dextrin. With the remaining sugars the results varied considerably.

2. Cultures proving non-virulent and producing acid with

some of the sugars, especially glucose and dextrin, were regarded as non-virulent diphtheria bacilli.

3. The sugar tests were found of most value in studying cultures from the eye and in dealing with D_2 types. Short, solid types proving non-virulent for pigs and producing no acid with any of the sugars were regarded as cultures of Hofmann's bacillus. Non-virulent cultures producing acid with saccharose were regarded as cultures of *B. xerosis*.

4. In routine work the sugar-tests are of distinct value when used in conjunction with tests for virulence. The short, solid D_2 type should not be confounded with Hofmann's bacillus, and since both may be non-virulent the sugar-tests alone can differentiate.

DISCUSSION.

Although great advances have been made in the diagnosis and treatment of diphtheria, yet the fact remains that the disease occurs with almost unabated frequency. Before the morbidity can be effectually lowered there must be hearty co-operation between practitioners and public health officials. There will always be the atypical and unrecognized cases to be dealt with—cases capable of spreading the disease—but with the conscientious use of the culture outfit a number of these may be recognized. The ability of the average practitioner to diagnose diphtheria in its various manifestations, and especially to differentiate the infection from similar clinical conditions, is as a rule overrated. Many insist upon the typical test-book picture of diphtheria before making a diagnosis, others depend too much upon the results of bacteriological examination. Diphtheria of the nose is especially likely to be confusing.

The physician should realize his limitations in making correct differential diagnoses and properly culture all "suspicious" cases. He should be thoroughly acquainted with the etiology of diphtheria and recognize the existence of "carriers" of dangerous bacilli and the possibility of others contracting the disease. He should know likewise that virulent bacilli may persist in the tissues of his patient for a long time although the latter feels and looks perfectly well. And furthermore he should take pains to explain these

factors to his patient or the family in order to protect himself in case a prolonged quarantine is necessary. Too many practitioners commit themselves to a diagnosis in a doubtful case before the results of cultures are ascertained, and consequently are often required to change their diagnosis. Others promise quick recovery and relief from quarantine when they should know they are unable to foretell the length of quarantine if such is to be regulated by culture.

One negative culture from a throat showing extensive exudate should not be accepted as evidence of absence of diphtheria. Clinical judgment should rule and antitoxin be administered. A second culture should then be made. No medical man is justified in withholding antitoxin 24 hours to await the report of a culture. The fear of anaphylaxis in case the patient has received serum on a previous occasion is overdrawn and too prominent in the minds of medical men. Cultures should be taken, and taken repeatedly, from persons in contact with the patient, if sore throat or nasal discharge develops. A hospital ward offers the greatest practical difficulty because where a large number are cultured, a few are most likely to show the presence of diphtheria-like bacilli which may or may not be virulent. Such persons should be removed for a day or so until further bacteriological examinations are made. In the meantime a sharp lookout is maintained for clinical developments. The wholesale taking of cultures, especially of those not in immediate relation with the patient, is unnecessary.

For the proper management of diphtheria according to bacteriological examinations an effort must be made to differentiate between the harmful and harmless bacilli. For instance, a "carrier" may be quarantined forever if guided by the fact that the presence of a diphtheria-like bacillus means diphtheria. The medical attendant must be convinced of the value of bacteriological examinations and of the earnest efforts of the bacteriologist to work with him both in the interests of his patient and for the public welfare. The practitioner must be taught not to be afraid to report all of his cases and to culture faithfully. If, however, bacteriological examinations serve to give unnecessary embarrassment to the practitioner by quarantining "carrier" cases, or by

maintaining a quarantine unnecessarily long after convalescence as a result of conscientious efforts on the part of the latter to do his duty in taking cultures, he is not likely to give his best co-operation, and without this no real good is possible.

We advise the following scheme, modified after Wesbrook, of bacteriological diagnosis and management of diphtheria:

1. Hold the granular types, A, B, C, D, and E, as significant, regardless of the clinical condition or history of the patient or of the presence or absence of other types. Cultures of these types from "carriers" or from persons running a prolonged list of positive cultures should be tested for virulence.

2. Regard the barred types, A_1, B_1, C_1, and D_1, as "doubtful" unless granular bacilli are also present when a positive diagnosis is given.

3. Hold the solid types, A_2, B_2, C_2, and D_2, as "doubtful" and request another culture. If granular bacilli are present a positive diagnosis is given. If subsequent cultures show the same solid types and if the patient is free of clinical evidences of diphtheria they may be regarded as negligible. It is in dealing with these types that we feel the greatest need for clinical co-operation, because clinical diphtheria may in exceptional instances be caused by these bacilli, although in the majority of cases the D_2 type is a negligible organism.

4. Regard E_1, E_2, F, G, F_1, and F_2, as negligible.

5. Test the virulence of all cultures of solid types of bacilli after the patient has recovered and is being quarantined over a long period of time by positive cultures. These inoculation tests are especially indicated with cultures from the nose and ear.

If the above method is employed by the bacteriologist and if three successive negative cultures are required instead of two before quarantine is lifted, combined with the judicious use of the animal inoculation test in suitable cases, we feel that the bacteriological diagnosis and management of diphtheria will be efficient and satisfactory to all.

A METHOD OF OBTAINING CULTURES FROM THE DUODENUM OF INFANTS.*†

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Ever since Escherich[1] published his classic monograph on the flora of the intestine during infancy, this field has been a favorite one for bacteriologists. They have, however, found themselves hampered not only by the difficulties attendant on growing intestinal bacteria, but by the difficulty of isolating pathogenic organisms from the multitude of saprophytic bacteria which flourish in the lower intestinal tract. Bearing this in mind, it seemed as if the duodenal catheter, devised primarily for the purpose of the diagnosis and treatment of pyloric stenosis, might be of value in this connection and afford a new method for the approach of this subject. This instrument is merely a soft rubber Nelaton catheter No. 14 or No. 15 (F) and marked in an appropriate manner. It can be inserted through the pyloric sphincter and into the duodenum in infants, from the time of birth up to the age of two years, with a facility comparable to the passage of the ordinary stomach-tube in the adult. It seems hardly necessary to outline the technic of this procedure, in view of the fact that a previous article[2] describes it in detail, including radiographs which clearly demonstrate the presence of the catheter within the lumen of the upper intestine.

For the purpose of bacterial investigation this duodenal catheter was sterilized and then capped at the end with a gelatin capsule, such as the apothecary frequently makes use of in dispensing drugs. This capsule had been sterilized by dry heat at 160° C. and then, by means of sterile forceps, was slipped over the eye at the end of the catheter, and fastened by slightly moistening with

* Received for publication May 7, 1912.

† Presented at the Annual Meeting of the Association of American Bacteriologists and Pathologists, April 6, 1912.

[1] *Die Darmbakterien des Säuglings*, 1886.

[2] *Am. Jour. Dis. Children*, 1912, 3, p. 133.

sterile water. The catheter thus prepared was passed in the usual way into the mouth and the stomach, and then introduced gently through the pylorus into the duodenum. In general the pylorus can be traversed by this means within three to five minutes. It was found that the capsule, which had become hardened, due to the heat of sterilization, did not melt before it reached the duodenum; this was readily ascertained by means of aspiration, for as soon as the end of the catheter is open, a drop or two of gelatin can be aspirated, whereas as long as it is closed, a marked sense of resistance to aspiration is encountered. By means of this technic it is possible to obtain the contents of the duodenum and of the jejunum, by the aid of antiperistalsis, with but very slight contamination from the mouth or the stomach. It is probable that a slight degree of contamination generally results, but, judged from what follows, this could not have occurred to a considerable degree. As an additional safeguard, the gelatin covering the eye of the catheter, which might constitute a source of contamination, was not sucked up with the first aspiration, but, on the contrary, was blown off the end of the tube.

The first tests were made upon new-born babies, a few hours old, before they had taken any food whatsoever. This group consisted of four cases. Very few bacteria were found in the stomach and in the duodenum. Only one to three bacteria to a platinum loop of the fluid. These were staphylococci, molds, gram-positive and, less often, gram-negative bacilli. No colon bacilli were found.

Next, 11 babies were tested during the first week of life. In each case a test of the stomach contents was first made, then one of the duodenal contents, the tests being carried out when the stomach was comparatively empty. Here, again, very few bacteria were found in the duodenum, and the same varieties were cultivated as in the new-born babies. As was to be anticipated, there was a general concordance between the bacteria of the stomach and those found in the upper intestine. In only one case of this series was the colon bacillus, or, indeed, any gas-producing organism, obtained; in these tests 0.3–0.5 c.c. of intestinal contents was cultivated. For comparison, a test of the mecomium was

made for the presence of the colon bacillus. The earliest that this organism was met with was in the case of an infant 12 hours old. Generally it was not found in the mecomium. In this connection we may call attention to the work of Escherich, of Moro, and of Herter, who found the colon bacillus quite frequently in the mecomium, and therefore suggested that this bacillus first gains access to the gastro-intestinal tract by way of the rectum. The most common bacterium in the upper intestine during this period of life was the staphylococcus, in the stomach as well as in the duodenum. The bacteria seemed to be almost entirely of the facultative variety, for anaerobic cultures failed to show other organisms than those grown under aerobic conditions. It was found that the presence or absence of free hydrochloric acid in the stomach, or of a small or large quantity of bile in the intestine, did not play a constant rôle as regards the number of bacteria obtained. In tests made upon somewhat older infants, two to six weeks of age, the colon bacillus was almost regularly cultivated from the duodenum, and with a less degree of constancy from the stomach. However, these various tests are not considered conclusive and are given, not so much with the idea of furnishing a definite qualitative and quantitative test of the normal flora at this period of life, for we realize that they are insufficient in number to serve this purpose, but rather to show how this method may be made use of in this connection. The number of bacteria cultivated from the stomach was found almost always to be in excess of that met with in the duodenum, which, in itself, furnishes a conclusive argument that the duodenal cultures were not merely contaminations from the mouth and the stomach. The number of bacteria in the duodenal contents was at no time great. During the first few days of life hardly more were found than in the tests made before the infant had ever nursed. At this period and a little later, 25 to 100 bacteria were usually grown from a loop of intestinal contents, and even in infants several weeks of age not more than 100 to 200 organisms were cultivated per platinum loop. In the case of bottle-fed babies the number of bacteria was regularly higher. This comparative sterility of the duodenum was not surprising in view of the work of Kohlbrugge and others on the

auto-sterilization of the intestines. Some years ago bacteriological tests of the contents of the intestine of rabbits, which we undertook in connection with a study of antiperistalsis, also demonstrated this fact decisively.

In addition to these general considerations, we should like to mention two specific instances where these tests seemed to be of clinical interest. One was the case of an infant a week old, who had gastro-intestinal symptoms, coated tongue, some vomiting, and green stools; in this instance repeated tests showed a large number of staphylococci in the stomach, and a still larger number in the duodenum.

The opportunity presented itself of passing the catheter upon a baby 22 months old, suffering from typhoid fever in the beginning of the third week, and giving a positive Widal reaction. A small amount of bile-containing fluid was aspirated from the duodenum, and from this fluid typhoid bacilli were cultivated in very large numbers. The marked success in this instance strengthened the idea that this simple procedure might be of value in this connection in the diagnosis of typhoid carriers. Although this catheter cannot be used upon adults, as it does not readily pass through the pylorus where the stomach is large and the sphincter is resistant, a duodenal tube, as devised by Gross or by Einhorn, which in the course of a few hours is carried into the duodenum by means of peristalsis, might well be used for this purpose. This tube is very thin and causes very little inconvenience, as I have had opportunity to observe. The advantages of a duodenal test for typhoid bacilli, over a test of the stool, is considerable, as the duodenum is the estuary for typhoid bacilli. In the intestine by far the greatest number of typhoid bacilli are found in the duodenum and the jejunum, as could be presupposed when we consider that they are in the blood at the very onset of the disease, that they are partly excreted from the blood into the bile, and having multiplied in this fluid are poured into the upper intestinal tract. In this connection we should also remember that one of the very last organs of the body that they forsake is the gall bladder, which is the favorite nidus of this bacillus within the body of the typhoid carrier (Chiari). A clear and conclusive conception of the inci-

dence of the typhoid bacillus in the gall bladder and the various levels of the intestinal tract may be obtained from the table of Gaehtgens[1] which is appended (Table 1). From this table, which gives the results of cultures taken post mortem in cases of typhoid and typhoid carriers, it is clear that the typhoid bacilli decrease in numbers in the intestinal tract as we proceed downward from the duodenal opening of the bile duct to the rectum, and that they are to be found most constantly in the gall bladder.

This idea of cultivating typhoid bacilli from the duodenal contents, rather than from the stool, is not entirely original. Some years ago Weber made an attempt of this kind on account of the difficulty which had been experienced by others in cultivating typhoid bacilli from the stools of carriers. Kayser,[2] of the Kaiserliche Gesundheitsamt, found in a series of cases, that three repeated examinations at long intervals failed to show typhoid bacilli, where later tests proved the individuals to be carriers, and therefore recommended as a safeguard that tests of carriers should be carried out every two or three months for a period of a year. In view of the inadequacy of stool examinations, Weber[3] made use of Volhard's oil breakfast, which consists in giving about 200 c.c. of olive oil by mouth to incite a regurgitation of the duodenal contents into the stomach. He then attempted to cultivate typhoid bacilli from the stomach contents. In three patients, two of whom were typhoid carriers and one a paratyphoid carrier, a very large number of typhoid colonies were thus obtained, whereas they were cultivated but sparsely from the stool. Wherever bile was found in the stomach, typhoid bacteria were likewise found in large numbers. The only difficulty was that the duodenal contents could not always be obtained by this means.

We wish in this paper merely to present for consideration a method for the diagnosis and the study of bacterial conditions of the intestine. It is possible that this technic may also be of value in connection with diseases of known, or unknown origin, associated with animal parasites, such as, for instance, amebic infection of the intestine or of the liver. It may also have an application

[1] *Berl. klin. Wchnschr.* 1912, 49, p. 296.

[2] *Arb. a. d. k. Gsndhtamte.,* ——— 1906, 24, p. 173.

[3] *München med. Wchnschr.,* 1908, 55, p. 2443.

in the study of the influence of the various food-stuffs upon the bacterial flora of the intestine. However, in addition to its practical value in the diagnosis of the typhoid carrier, at present its main field of usefulness would seem to be in the study of dysentery and enteritis, diseases which play such an important rôle in the morbidity and mortality of early life, and which it is difficult to investigate satisfactorily by the aid of cultures from the stool on account of the vast number of contaminating bacteria. This difficulty is not encountered in cultures taken from the upper part of the intestinal tract.

TABLE I.

POST MORTEM CULTURES OF TYPHOID CASES AND CARRIERS, SHOWING INCIDENCE OF B. TYPHOSUS IN GALL BLADDER AND VARIOUS LEVELS OF THE INTESTINE.

	Gall Bladder		Duodenum		Jejunum		Ileum		Colon		Rectum	
	+	−	+	−	+	−	+	−	+	−	+	−
1st Week....	..	..	..	1	..	1	..	1	..	..	..	1
2d "	2	1	2	..	2	2	1	2	1	1	..	2
3d "	6	..	2	2	2	2	2	3	1	7	..	..
4th "	2	..	2	1	3	2	1	3	..	2	..	4
5th "	2	..	..	..	1	1	..	1	1	1	..	..
6th "	..	..	..	..	..	..	..	..	..	..	..	..
7th "	2	..	..	..	1	1	..	2	..	2	..	..
8th "	1	..	..	..	1	..	..	..	..	1	..	..
Total.....	15	1	6	4	10	9	4	12	3	14	0	7
Per cent...	96		60		53		27		18		0	

+ = Typhoid bac.
− = No typhoid bac.

CUTANEOUS ALLERGY IN GONOCOCCAL INFECTIONS.*

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The inoculation of the gonococcus into the body of man or animals causes changes in the blood which have been demonstrated by studies of the specific agglutinins, precipitins, and opsonins. More recently the antibody content of the serum has been estimated by means of complement fixation, using preparations of the gonococcus as antigen. By all these methods, the variations in the course of gonococcal infection may be more or less satisfactorily followed, and, in the case of the opsonins at least, the curve of immunity may be correlated with the changes in the clinical course of the disease.

Certain reactions, also, follow the subcutaneous inoculation of suspensions of killed gonococci in patients suffering from gonococcal infection. These reactions are characteristic[1] and are analogous to those seen after the injection of tuberculin, mallein, etc., in the respective diseases. At the site of the inoculation there appears after a few hours, an area of redness, swelling, and tenderness, from 2 cm. to 6 cm. or more in diameter, depending upon the amount of the suspension inoculated. Sometimes the whole upper arm becomes swollen and painful. After 24 to 48 hours the reaction subsides. There is frequently an increase in pain and sometimes swelling in the affected joints or other localizations, together with general symptoms of malaise, headache, and fever. An increase in leukocytosis may occur.

The degree of the reaction varies in different cases with the extent of the infection and the general condition of the patient. In gonococcal arthritis in which active symptoms have been absent for some time, a subcutaneous inoculation may produce little more than a local reaction at the site of the injection. In chronic types of arthritis in which there have been repeated severe attacks of the

* Received for publication May 23, 1912.

[1] *Jour. Infect. Dis.*, 1908, 5, p. 270.

infection, the reaction may be very slight or absent. These cases often do badly under vaccine treatment. In cases in which repeated inoculations are made for therapeutic purposes, the reactions usually grow less with successive doses of the same size. Patients who have received a number of inoculations of moderate doses, without exhibiting more than a slight reaction, occasionally show a pronounced reaction after an inoculation of the same size, given in the same way as previous doses.

Ocular reactions following inoculations are sometimes observed in cases in which iritis has previously been present. A man of 35 who had suffered from arthritis and iritis six years previously complained of pain in the joints of the spine and ankles. There had been no recent active trouble in the eye. He was given a subcutaneous inoculation of 100 million gonococci for diagnostic purposes. Twenty-four hours later the previously affected eye became painful: photophobia and some conjunctival injection were present. The other eye showed no change. The symptoms subsided after 24 hours. Reactions characterized by increased inflammation, or by effusions in the eye, are not infrequent after therapeutic inoculations of vaccine in active gonococcal iritis. In a number of cases, however, such reactions have been entirely absent, even after large doses of vaccine.

The degree of reaction depends not only on the number of gonococci inoculated, but also on the source and age of the cultures from which the vaccine is prepared. Recently isolated cultures (two weeks to three months, fifth to 40th generation) furnished a vaccine 10 times as strong as a culture which had been grown in the laboratory for two years. This variability in the activity of vaccines may explain the differences of opinion as to the optimum therapeutic dose of gonococcal vaccine.

The reaction has a diagnostic value in obscure cases of arthritis, and its use has also been suggested for the determination of the cure of local gonococcal infections. Bruck[1] has observed the reaction in epididymitis, and Reiter[2] in pelvic infections in women, and in arthritis.

[1] *Deutsche med. Wchnschr.*, 1909, 35, p. 470.

[2] *Ztschr. f. Geburtsh. u. Kinderh.*, 1911, 68, p. 471.

CUTANEOUS ALLERGY.

The cutaneous reaction to injections of preparations of the gonococcus has been studied by several workers. Bruck[1] obtained a "cutireaction" by intradermal inoculation of gonococcal vaccines. Köhler[2] inoculated, by the method of von Pirquet, vaccines prepared from streptococcus, gonococcus, colon bacillus, etc., and was able to demonstrate the specificity of the reaction in cases of gonococcal infection.

In recent experiments with glycerin extracts of the gonococcus, I have found that a well defined cutaneous reaction, similar to the cutaneous tuberculin reaction, occurs in cases of gonococcal infection, when the extract is introduced into the skin by the method of von Pirquet, and that the course of the immunity curve in a given case may be traced from day to day by noting the degree of the reaction after successive inoculations. These curves are similar to those obtained by daily estimations of the opsonic index, and their fluctuations may be correlated with the clinical changes observed in the patient. In recently infected cases, the cutaneous allergy is seen to increase steadily from day to day.

PREPARATION OF MATERIAL FOR TESTS OF CUTANEOUS ALLERGY.

Cultures of strains of the gonococcus were grown on ascites agar for 24 hours, the growth removed with sterile distilled water, 0.5 per cent phenol added, and the suspensions placed at 37° C. for 48 hours. The fluid was then heated to 60° C. for two hours, glycerin added, and the mixture evaporated in a current of warm air. The final product was a clear yellowish fluid of the consistence of glycerin. In the following discussion it will be referred to as "gonococcin" in conformity with the terminology of other similar products, such as tuberculin, leprolin, tricophytin, luetin, etc.

The technic briefly described has been modified in various ways in the course of the work, without essentially changing the results. Human blood agar as a culture medium was substituted for ascites agar without changing the action of the final product. No difference was noted in the products when normal salt solution was used

[1] *Loc. cit.*

[2] *Wien. klin. Wchnschr.*, 1911, 24, p. 1564.

in place of distilled water for making the suspensions, and the latter was adopted to avoid the possible irritant action of the salt in the concentrated product.

Material for control inoculation was prepared in precisely the same manner, using the washing from the same number of uninoculated culture tubes.

Cultures from five strains of gonococci were combined in the antigen used in most of these cutaneous tests. Experiments with antigens made from single strains showed that in some instances the reaction from one of the strains was less than that from the others. The strains used were derived from cases of arthritis and urethritis. In a case of vaginitis and ophthalmia an autogenous antigen gave the same degree of reaction as did the stock antigen of three combined strains.

Cultures of the gonococcus from 24-hour blood agar transfers were planted on large tubes (2.5 cm.×15 cm.) of slanted ascites agar and incubated for 24 hours. Care was taken that the seeding of the surface of the slants was uniform, and drying of the surface was prevented by cork stoppers in the tubes. The suspension obtained from six tubes was used in the preparation of 1 c.c. of the final product.

A somewhat more exact method of preparing the antigen was attempted by evaporating a suspension to dryness, and then taking up the weighed powdered residue in glycerin. This residue necessarily contained some soluble products from the culture medium, in addition to substances derived from the gonococcus. Serial dilutions of this extract were prepared, and the reactions obtained with them compared with the reactions from the extract prepared by the first method. It was found that an extract containing 0.1 gm. of the powdered residue in 1 c.c. of glycerin gave approximately the same degree of reaction as did the extract prepared by the first method. The reactions were progressively less, the greater the dilution of the gonococcin. A dilution of the extract containing 0.001 gm. per c.c. of glycerin gave a faint reaction in allergic individuals.

The time allowed for autolysis of the suspensions of the gonococcus seemed to affect the strength of the gonococcin. An antigen

prepared from a suspension of gonococci heated to 60° C. immediately after removal of the organisms from the surface of the culture medium did not give quite as strong reactions in gonococcal cases as did an antigen prepared from the same suspension and kept at 37° C. for 48 hours before heating to 60° C. In cases in which the cutaneous allergy was highly developed, the difference between the two antigens was absent.

A suspension of gonococci was evaporated to dryness, and the residue extracted for 48 hours with 95 per cent alcohol. An antigen prepared by evaporating the alcoholic extract and adding a suitable amount of glycerin gave only a slight reaction in allergic persons. The residue remaining after extraction by alcohol was extracted with water for 24 hours. An antigen prepared from this extract gave a good reaction in susceptible individuals.

METHOD OF INOCULATION.

The preparations of gonococcin and the control extract were kept in a battery of cork-stoppered vials. A separate needle was used for each preparation. When not in use, the needles were kept in vials of alcohol, each corresponding to a vial of antigen. In this way each needle was sterile, and used only for its own antigen.

Inoculations were made by gently rotating the point of the needle with a minute drop of antigen attached on the skin of the arm. Care was taken to avoid the puncture of capillaries. In all cases a series of at least three inoculations and one control was made, care being taken to make the inoculations as nearly uniform as possible.

THE CUTANEOUS REACTION.

The cutaneous reaction after the inoculation of gonococcin in infected individuals does not differ essentially from that following the inoculation of other antigens such as tuberculin. Within a few minutes small wheals may appear about the point of puncture of both antigen and control. After a few hours a papule is formed, with a surrounding area of hyperemia, and in 24 hours the maximum reaction is usually obtained. On the second day the hyperemia is less, and by the third day has usually disappeared. The papules remain for several days, and in pronounced reactions may

be visible seven to 10 days after inoculation. Delayed reactions have not been observed.

The control inoculation shows only a minute point at the site of trauma.

The degree of the reaction in gonococcal infections varies with the extent of the infection, and from time to time in the same case. Typical reactions in gonococcal arthritis or epididymitis show a papule 3 to 5 mm. in diameter, with a surrounding hyperemia of 7 to 10 mm. or more. Reactions of less degree were frequently observed in known gonococcal infections either early in the infection or later in the course of the disease.

Normal adults in whom previous gonococcal infection was excluded showed a lesion rarely more than 1–2 mm. in total diameter. Often the points of puncture showed nothing more than the control. Adults suffering from other diseases, such as typhoid fever, tuberculosis of bones and pleura, staphylococcus infections (furunculosis, osteomyelitis), leukemia, lobar pneumonia, etc., in whom previous gonococcal infection was excluded gave no more reaction than the average normal adult.

For the purposes of discussion the reactions obtained in this study have been classified as positive when over 5 mm. in total diameter, as negative when 3 mm. or less, and doubtful when the papule was small and the hyperemia indefinite or less than 5 mm. in diameter. This division, though arbitrary, is based on tests in a large number of normal and infected individuals, using antigens prepared in the manner indicated, and offers a convenient solution of the difficulty of discussing the differences in cutaneous reactivity of the cases studied.

In children in whom there is no history of gonococcal infection, about 50 per cent give no more reaction with gonococcin than with the control. In a number of the remainder a small area of hyperemia 2–3 mm. in diameter is obtained, and in a few, larger areas up to 5 mm., rarely 7 mm., are seen. This increased cutaneous sensitiveness in children has been observed in other infections, and has been explained by some on the ground that in children the amount of normal antibodies is proportionately larger than in adults. In children as in adults the increase in reactivity may be

observed in successive tests during the course of the infection, and a child whose skin gives a reaction of 1–2 mm. at the beginning of the disease, after two or three weeks, may give a pronounced reaction of 7–8 mm. The possible influence of infections of the nasal passages of these children by organisms, closely allied to the gonococcus, such as *M. catarrhalis*, must also be considered. The question of a group reaction will be discussed later.

Observations on patients with gonococcal infections soon demonstrated that while the degree of reaction may remain fairly constant from day to day, it is much more frequent for the reaction to vary in intensity from time to time.

The diameter in millimeters of the zone of hyperemia surrounding each papule was recorded 24 hours after inoculation. The results from three simultaneous inoculations were averaged, and this average used in plotting curves to show the fluctuations of the reaction in a given case. As a rule there was no marked difference between the three inoculations.

The daily inoculation of even minute quantities of the preparations may have produced a slight sensitization, but this was apparently very small compared to the much larger influence of the infection in the body.

In cases in which it was desired to study the development of immunity in infections unmodified by treatment, tests were made at intervals of three or four days.

The danger of confusion in the reactions through the production of antibodies by the albuminous constituents of the culture media was obviated by the use of the control inoculation.

The hyperemia resulting from the occasional infection of the point of inoculation of the antigen or control must be distinguished from a true reaction. Such infections usually show a minute white central area of pus formation. Careful preliminary cleansing of the skin with alcohol, and the making of multiple inoculations of each antigen will usually obviate this difficulty.

THE DEVELOPMENT OF CUTANEOUS ALLERGY.

Several cases of recent vaginitis in children in which the date of first discharge was known were tested at intervals of four to five

days. In all, the reaction in the first week was negative (i.e., there was no more reaction than occurs in normal individuals). In the second and third weeks, the papules became slightly larger, and by the fourth week pronounced papules with surrounding hyperemia were noted.

In epididymitis accompanying gonorrhea, the reaction varied in the cases studied. In several, tested on the third or fourth day of the epididymitis, cutaneous reaction was pronounced. In

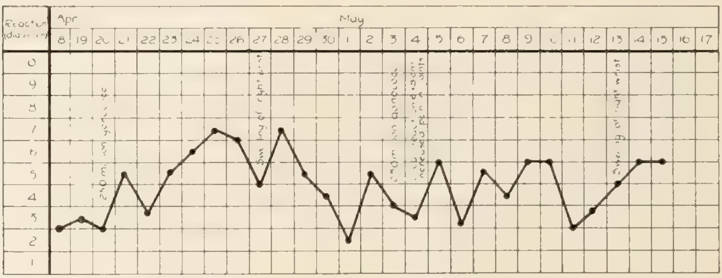

CHART I.—Cutaneous allergy with respect to gonococcal extracts in a man 20 years old; first attack of gonorrhea in September, 1910; recurrence in August, 1911; arthritis in October, 1911; no discharge at present; now suffers from tender heels and ankles, and recurrent hydrops of right wrist with 7 days' cycle. Injections of killed gonococci as shown on the chart.

two cases the reaction was faint on the second and third days, but by the fifth day, when the patient had begun to mend, the reaction became more definite.

In arthritis, the degree of the reaction varied greatly. In several cases of extensive arthritis, the reaction was negative or slight. Later, when improvement in general condition occurred, the cutaneous reaction became pronounced. In one case of extensive arthritis, in which improvement did not occur while under observation, a positive reaction was not obtained.

In infected individuals giving a weak cutaneous reaction, the reaction may become positive on a second test after the inoculation of gonococcus vaccine (Chart I[1]).

[1] The curves shown in these charts were constructed from successive determinations of the degree of cutaneous reaction following the inoculation of glycerin extracts of bacterial antigens. The degree of reaction was determined by recording the diameter in millimeters of the reactions obtained 24 hours after inoculation. In the gonococcal curves, each determination represents the average diameter of three simultaneous tests. The meningococcal curves were plotted from single determinations. Care was taken to make the inoculations as nearly as possible uniform in depth, and in the amount of antigen inoculated. The reactions obtained from day to day were measured, and the data recorded on slips of paper without

DURATION OF CUTANEOUS ALLERGY.

Several adults tested from one to two years after a single gonococcal infection gave positive reactions. A number of adults with a history of gonorrhea from 2 to 12 years previously gave

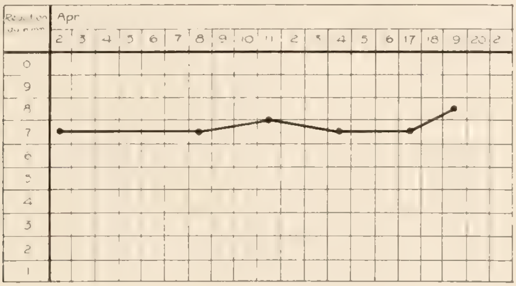

CHART 2.—Cutaneous allergy with respect to gonococcal extracts in a woman, 25 years old, who acquired gonorrhea one and one-half years ago; bilateral salpingitis. Panhysterectomy on April 3. Gonococci were found in wall of tube. Rapid recovery after operation.

reactions considerably more pronounced than the average normal adult with a definite negative history. Too much importance should not be attached to the results in this latter class of cases, as the possibility of a persistent gonococcal posterior urethritis or prostatitis was not excluded.

In arthritis the wave of immunity, as shown by cutaneous allergy, may rise and fall, even below the threshold of the cutaneous reaction, and it is probable that this oscillation occurs as well in other types of gonococcal infection.

A case of bilateral pyosalpinx (Chart 2) gave a positive reaction before operation, and after panhysterectomy the reaction remained at the same height till the patient passed out of observation three weeks later.

INFLUENCE OF OTHER INFECTIONS ON THE GONOCOCCIN REACTION.

Intercurrent diseases such as whooping cough, chicken-pox, and mumps, in children suffering from vaginitis, did not seem to

reference to previous figures, and later the accumulated results were plotted in curves. It is recognized that this method of recording results is open to criticism, particularly in the matter of small daily variations. The more pronounced variations, however, can hardly be attributed to errors in the methods of observation, and compare favorably in point of accuracy with the data obtained in laboratory experiments.

interfere with the gonococcin reaction. Three cases of gonococcal infection in persons with active syphilis gave unusually bright reactions.

TABLE 1.

	Reaction 5 mm. or More +	Reaction 3–4 mm. Indefinite ?	Reaction No Reaction or 2–3 mm. —
Gonococcal arthritis	11	2	2
Epididymitis	6	1	1
Old gonorrhea 1–20 years	3	6	4
Ophthalmia	2	0	0
Iritis	2	0	0
Salpingitis	5	2	3
Vaginitis, Series I	9	0	2
Series II	13	9	4
Series III	7	3	8
Series IV	8	3	8
Normals and other diseases			
Gonorrhea denied. No signs	1	1	16
Puerperal sepsis clinically not gonorrheal	0	0	6
Fibroids—old, cystic ovaries, and endometritis	0	0	5
Children—other diseases, Series I	2	7	9
Children—other diseases, Series II	1	9	12

Table 1 shows the proportion of positive, negative, and doubtful reactions obtained in some of the series of gonococcal infections, using the arbitrary classification described above. The cases are arranged in groups, according to wards, clinics, and hospitals. In the series of arthritis, the diagnosis was established with certainty. The negative and doubtful reactions were met with for the most part in patients with extensive arthritis and little clinical evidence of recuperative power. Certain of these severe cases showed first a negative, and later a positive reaction when improvement had set in.

The several series of vaginitis show considerable variations in the proportions of positive and negative cases. This may be due in part to the fact that some series comprised cases of longer standing than others, and in part to the fact that although certain of these children were in vaginitis wards, suffering from vaginitis, the gonococcus could not be demonstrated in the smears or in cultures. It is of course recognized that a single culture from the vagina negative for the gonococcus, in the presence of many other organisms, is by no means conclusive evidence that the gonococcus is not present, but when such cultures are repeatedly negative for gonococcus, and show at the same time diplococcoid forms of other

gram negative organisms, one is led to wonder whether these latter may not have been the intracellular gram negative organisms on

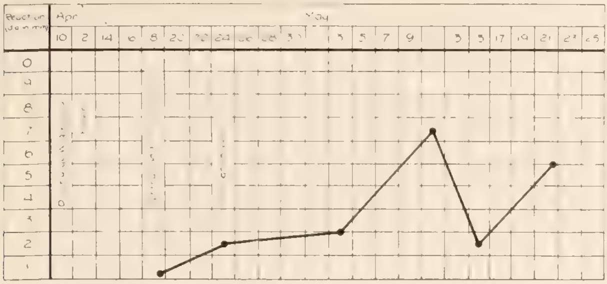

Chart 3.—Cutaneous allergy with respect to extracts of gonococci in a girl 3 years old, in whom gonococcal vaginitis with ophthalmia developed as indicated in the chart.

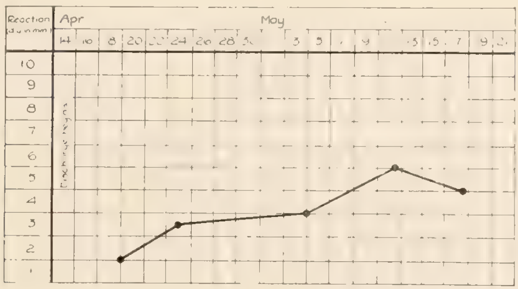

Chart 4.—Cutaneous allergy with respect to gonococcal extracts in gonorrheal vaginitis in a girl 2 years old.

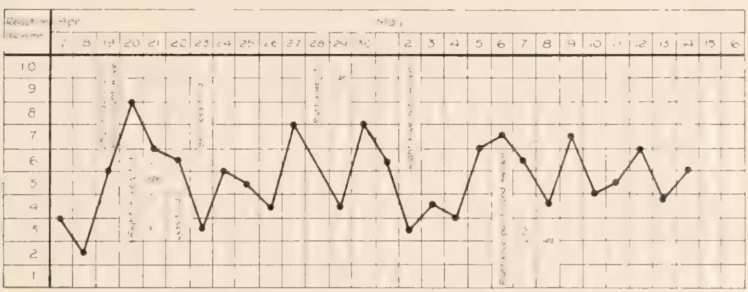

Chart 5.—Cutaneous allergy with respect to gonococcal extracts in recurrent hydrops of the right knee.

which the diagnosis of gonococcal vaginitis was made by the physician. Several cases of long-standing vaginitis which gave a

persistently negative cutaneous reaction were studied by means of repeated vaginal cultures, and the gonococcus was not found. The same methods and media gave good growths of the gonococcus from other cases including vaginitis and urethritis. It seems likely, therefore, that in some cases of vaginitis in specific wards the gonococcus is not present.

In testing adults who were supposedly free from gonococcal infection, several were found, including a case of alcoholic cirrhosis, and one of tuberculous pleurisy, who gave rather marked cutaneous reactions. Two of these were women in whom an inactive infection could not be excluded. In the case of alcoholic cirrhosis, the habits of life of the patient were such that gonococcal infection was at least possible.

The finding of occasional positive reactions in supposedly non-gonococcal cases and the failure of some cases of arthritis to give continuously a positive reaction, suggest the advisability of further series of tests, before absolute reliance is placed on the reaction in diagnosis.

In salpingitis of varying duration (6 months to 10 years) about 50 per cent gave positive reactions. In the remainder a faint or negative reaction was obtained. This latter group contained several old cases in which the record of operation showed extensive adhesions, cystic tubes, and ovaries without active pus formation. In one of the cases giving a positive reaction, large pus tubes were found, from the walls of which the gonococcus was obtained (Chart 2).

CUTANEOUS ALLERGY IN MENINGOCOCCAL INFECTIONS.

Four cases of epidemic meningitis were studied. A boy of two years, convalescent in the fourth week after the onset of the infection by the meningococcus, gave a positive reaction to gonococcin. There was no sign or history of any gonococcal infection.

A youth of 19 in whom there was no history or evidence of gonococcal infection was tested on the second day of a moderately severe meningitis, and gave a negative reaction. Tests on the sixth and eighth days were likewise negative. Four doses of anti-meningococcic serum had been given on the second to the fifth days,

and the patient was afebrile and convalescent on the sixth day. On the 13th day, the gonococcin produced a reaction of 3 to 4 mm. and a glycerin extract of the meningococcus isolated from the patient produced a reaction of 6 mm. The allergy continued to increase, and on the 17th day a reaction of 5 mm. was obtained with gonococcin, and one of 10 mm. with the preparation of the meningococcus (Chart 6).

A third case of meningitis in a man of 30 years was much more severe. He received two doses of serum of 30 c.c. each on the second

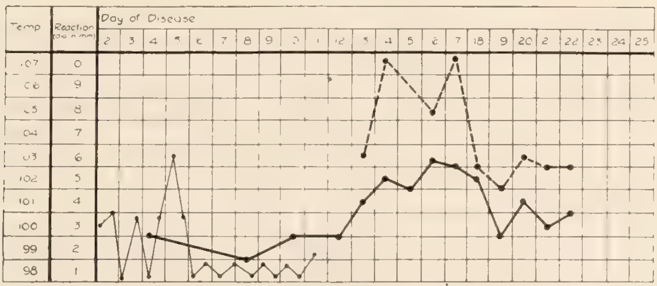

CHART 6.—Cutaneous allergy with respect to meningococcal and gonococcal extracts in a case of epidemic meningitis. Antimeningococcic serum (30 c.c.) given by lumbar puncture on second, third, fourth, and fifth days of illness. Fine line = Temperature. Heavy broken line = Reaction to meningococcal extracts. Heavy solid line = Reaction to gonococcal extracts.

to fifth days, became afebrile for five days and then the symptoms returned with high fever, headache, etc. On the 13th day the reactions to preparations of the gonococcin and meningococcus were still faint, and remained so until the 19th day, when they began to increase (Chart 7).

A fourth case of epidemic meningitis in a man who gave no history or evidence of gonococcal infection showed slightly positive reactions to gonococcin on the 12th day of the disease.

Patients with gonococcal infection who showed a marked cutaneous allergy were tested with the same antigens as the meningococcal cases described above. Definite reactions were obtained in the gonococcal infections with inoculations of a preparation of the meningococcus, though here the reaction was less than that obtained with the gonococcin.

GROUP REACTIONS.

These reactions with the gonococcus and meningococcus form a further demonstration of the close relationship which already has been shown by many workers by comparisons of the cultural characteristics of the two organisms, and by studies of their agglutinins and precipitins. It is possible that the micrococcus catarrhalis may be included in this group reaction, and infection by this organism may explain the occurrence of certain doubtful gonococcin reactions observed in persons supposedly free from gonococcal infection.

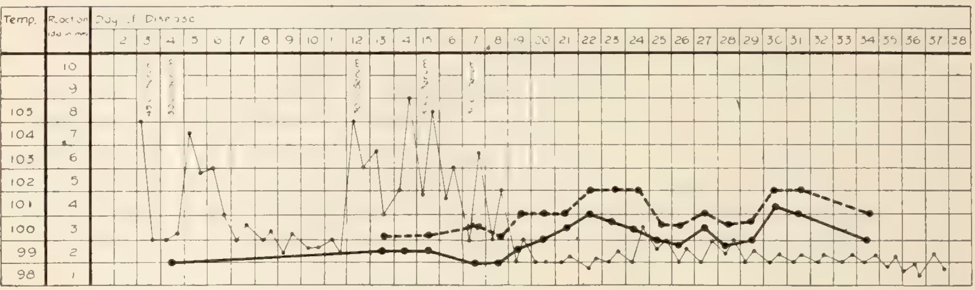

CHART 7.—Cutaneous allergy with respect to meningococcal and gonococcal extracts in a case of epidemic meningitis showing recrudescence of the disease on the 12th day and again on the 24th day, as shown by the temperature curve. Antimeningococcic serum given by lumbar puncture as indicated on the chart. The cutaneous allergy developed slowly and to a relatively slight degree. Fine line = Temperature. Heavy broken line = Reaction to meningococcal extracts. Heavy solid line = Reaction to gonococcal extracts.

Similar group reactions have been noted in studies of the interreactions of tuberculin and leprolin, and in the tricophytin reaction in which antigens prepared from tricophyton, microsporon, and achorion will all give reactions in persons infected with tricophyton, and the tricophytin will give reactions in persons suffering from favus.

EXPERIMENTAL ALLERGY IN MAN AND ANIMALS.

A man suffering from a fracture of the leg, in whom previous gonococcal infection was excluded by careful inquiry into the history and by examination, was found to give a negative cutaneous reaction to gonococcin. He was then given repeated subcutaneous injections of gonococcus vaccine and the subsequent cutaneous

reactions recorded (Chart 8). The maximum reactions were by no means as large as those often seen in infected persons, but compared to the reaction before inoculation, they showed a definite increase in cutaneous reactivity following the injections.

A large rabbit was given seven intraperitoneal injections of suspensions of gonococci grown on rabbit's blood agar. Five weeks after the last injection cutaneous tests were made with antigen and control. Slight papules developed after 24 hours at the site of inoculation of the antigens. The control inoculation showed no reaction. This test was repeated with the same result. A suspension from one blood agar tube of the same strain of gonococcus

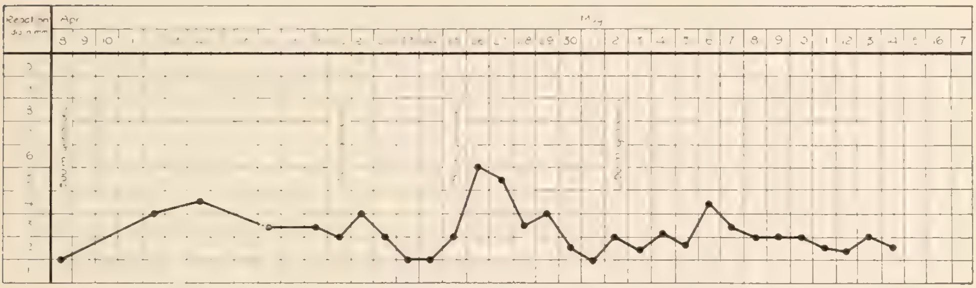

CHART 8.—Cutaneous allergy with respect to gonococcal extracts in a man without gonococcal infection but injected with killed gonococci as shown in chart.

was injected intravenously. A cutaneous test on this date gave papules 2 to 3 mm. in diameter. Two days later a reaction of 5 mm. with a definite hyperemia was obtained. Subsequent tests gave less marked reactions.

PASSIVE ALLERGY IN ANIMALS.

A number of normal guinea-pigs (250 gms.) and rabbits were shaved and cutaneous tests made. In all animals tested, no reaction beyond that due to trauma was obtained either with antigen or control.

Four of the pigs were then given intraperitoneal injections of 1 c.c. of commercial antigonococcus serum. Cutaneous tests after 24 and 48 hours were negative.

Two pigs were given 5 c.c. of serum intraperitoneally. Tests

made daily for the succeeding four days showed nothing beyond an occasional indefinite papule.

A pig was given 5 c.c. of concentrated antigonococcus serum. On the following day the cutaneous test was entirely negative, but on the second day a papule of 3 mm. was obtained, while the control was negative. On the third and fourth days the reaction was negative.

A half-grown rabbit was given 5 c.c. concentrated[1] antigonococcus serum intravenously. Slight papules appeared after cutaneous inoculation on the second, third and fourth days.

A rabbit was given 13 c.c. of fluid aspirated from the knee of a patient with gonococcal arthritis. Repeated tests on subsequent days were negative.

In these and similar experiments, the cutaneous reactivity was apparently slightly increased after the larger doses of concentrated serum, but the results were by no means marked.

SUMMARY.

The cutaneous inoculation of glycerin extracts of autolyzed gonococci in patients infected by the gonococcus produces a well defined reaction. This reaction is not usually obtained in normal persons, nor in those suffering from other infectious diseases.

In persons recently infected, the reaction is negative and increases gradually during the course of the disease.

In the more chronic forms of gonococcal infection, such as arthritis, the degree of the cutaneous reactivity varies from day to day, and these variations may be correlated with the changes in the clinical course of the disease.

Cases of severe infection, such as extensive arthritis, may give negative reactions. Later, when improvement has occurred, the reaction becomes positive.

In general, a positive reaction is obtained in patients with gonococcal infection at some time during the course of the disease.

In normal persons the gonococcin prepared in the manner

[1] Commercial antigonococcus serum was concentrated by Dr. P. G. Heinemann. The antibody content of the concentrated serum was estimated by Dr. Gatewood as about four times that of the original serum. *Jour. Infect. Dis.*, 1912, 10, p. 416.

described gives a cutaneous reaction rarely more than 2 to 3 mm. in diameter.

Occasionally in adults and somewhat more frequently in children fairly marked reactions are met with where previous gonococcal infection can be excluded. In these cases the normal antibodies may be increased to an unusual degree. It is possible that normal individuals may be found who will give reactions to antigens prepared from many pathogenic organisms. The possibility must also be borne in mind that infection by one organism may give rise to an increase in the proteolytic power of the serum for other organisms.

The cutaneous reactions obtained with meningococcal and gonococcal antigens suggest that we are dealing with a group reaction.

In diagnosis, a positive reaction is to be regarded as confirmatory evidence of gonococcal infection. Other infections, such as those by the meningococcus or *M. catarrhalis*, which may give rise to a group reaction, must be excluded. The clinical value of the reaction must be determined by further tests, and its limitations defined by a study of many groups of cases.

FURTHER STUDIES OF THE TOXIC SUBSTANCES OBTAINABLE FROM PNEUMOCOCCI.*

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Recently I have shown[1] that the appearance and then the disappearance of toxic substances in suspensions in salt solution of pneumococci and other bacteria, when kept at 37° C., are associated with proteolysis. These toxic substances, as measured in the guinea-pig, are very similar, and it makes no difference whether they are obtained by autolysis, by the action of normal or of immune serum, or by leukocytes *in vitro*. The symptoms caused are the same as those that appear in sensitized animals on reinjection of the unautolyzed extracts.

In the following I wish to present the results of further experiments on the toxic substances from pneumococci; on the conditions under which they appear and disappear in extracts and autolysates, in normal and immune serum mixtures, and in pneumococcus exudates; and on the relation they have to immunity in pneumococcus infections, and to anaphylaxis.

THE RELATIVE TOXICITY OF AUTOLYTIC EXTRACTS OF VIRULENT PNEUMOCOCCI FOR NORMAL AND SENSITIZED GUINEA-PIGS.

Although the difference in the behavior of normal and sensitized pigs to various pneumococcus extracts is mentioned in a previous paper a closer study of this point was thought worth while.

The pneumococci used in the experiment in Table 1 were grown in heated ascites-dextrose (0.2 per cent) meat-broth for 24 hours, washed once, and then suspended in salt solution so that 1 c.c. of the suspension contained approximately two and a half billion cocci. The virulence of the strain was such that one-half of the surface growth of a blood agar slant intraperitoneally killed a guinea-pig weighing 200 gms. in 16 hours. Disintegration of

* Received for publication May 25, 1912.

[1] *Jour. Infect. Dis.*, 1911, 9, p. 190; 1912, 10, p. 113.

pneumococci is directly proportional to the amount of proteolysis as indicated by formol titration.

The rotatory activity of this extract diminished and the amino nitrogen increased as the toxicity appeared and then disappeared. The results in Table 1 indicate strongly that the undigested pneumococcus protein is rapidly split into toxic material in the previously sensitized pig, and that the pneumococcus contains a ferment which splits its protein into similar cleavage products at a slower rate *in vitro* so that it becomes highly toxic for normal guinea-pigs and only slightly or not at all toxic to sensitized pigs. It seems as if the toxic material is rapidly split beyond the toxic stage in the sensitized animal. When the toxicity of clear pneumococcus

TABLE 1.

The Relative Toxicity of Autolytic Extracts of Virulent Pneumococci for Normal and Sensitized Guinea-Pigs.

Material Injected	Formol Titration	Levorotation in Minutes	Toxicity—7 c.c. Injected into Jugular Vein of Guinea-Pigs Weighing 200–225 Gms.	
			Normal	Sensitized
Virulent pneumococcus extract prepared at 37° C. for 5 hours	0.2 c.c. 0.5 c.c.	.. ..	No immediate symptoms. Death in 10 days	Death in 3 minutes from typical symptoms
Virulent pneumococcus extract prepared at 37° C. for 72 hours	0.3 c.c. 0.65 c.c.	12′ ..	Death in 2 minutes from typical symptoms	Slight symptoms, remained well
Virulent pneumococcus extract prepared at 37° C. for 5 days	0.3 c.c. 0.85 c.c.	2′ ..	No symptoms, remained permanently well	No symptoms, remained permanently well

extracts for normal pigs first appears the toxicity for sensitized pigs is greater than when the toxicity for normal pigs is at its height or is diminishing. Here the sensitized pigs often show no noticeable symptoms. Moreover, pneumococcus suspensions which have become toxic for normal pigs may be even more toxic for sensitized pigs. The undigested protein held by the partially autolyzed pneumococci must be the source of the toxic material. After proteolysis has gone still farther the toxicity for both normal and sensitized pigs disappears. The cause of the late death (10 days) of guinea-pigs (shown in Table 1, and observed many times before), which have received a larger dose of pneumococcus extract or suspension of pneumococci before the toxic stage is reached, is not

so clear; possibly it is due to sensitization so that undigested pneumococcus protein stored in the tissues is rapidly split into toxic material. The quantity of the acutely toxic substance in the circulation may not be great enough to provoke bronchial spasm. The late death is not observed in animals injected with autolysates which have become non-toxic. This explanation is in harmony with the work of Vaughan on egg white.

In this connection it should be stated that difference in the behavior of normal and sensitized pigs has been observed also with pneumococcus extracts which have been made toxic with immune serum and normal serum, with normal serum only, and with leukocytes; and also with salt solution extracts of pneumococcus exudates.

THE EFFECT OF NORMAL AND IMMUNE (SENSITIZED) GUINEA-PIG SERUM ON THE TOXICITY AND ROTATORY POWER OF PNEUMOCOCCUS EXTRACTS.

Extract 276, used in the experiments in Table 2, was prepared by suspending virulent pneumococci in NaCl solution for 24 hours

TABLE 2.

Effect of Serum on Pneumococcus Extracts.

Mixtures. 7 c.c. Injected into Normal Guinea-Pigs Weighing 200 Gms.	Levorotation and Toxicity			
	10 Minutes	2 Hours	4 Hours	24 Hours
Extract 276, 3 parts. Normal guinea-pig serum 1 part	1° 45′ No symptoms	1° 52′ No symptoms	1° 43′ No symptoms	1° 25′ Definite symptoms
Extract 276, 3 parts. *Immune guinea-pig serum, 1 part	2° 17′ Slight symptoms	2° 4′ Death in 4 minutes	2° 5′ Severe symptoms	1° 35′ No symptoms
NaCl sol., 3 parts. Normal guinea-pig serum, 1 part	2° 6′ No symptoms	2° 4′ No symptoms		
NaCl sol., 3 parts. Guinea-pig serum, 1 part	2° 9′ No symptoms	2° 4′ No symptoms		

* The guinea-pigs had been injected on two occasions with heat-killed virulent pneumococci.

at 37° C., ether being added and allowed to evaporate through the cotton plugs, and keeping for 48 hours in the ice-chest. Autolysis had gone on so that 7 c.c. produced severe but not fatal symptoms in a normal pig, and no symptoms in a sensitized pig of the same weight. The polariscopic reading of the extract at the time of the experiment was 5′ levorotation. The polariscopic readings were

made in a 10 cm. tube. While a constant relation between the rotatory power of mixtures of pneumococcus extracts and normal and immune serum, and their toxicity, cannot be made out, the appearance and disappearance of the toxicity are always associated with a diminished rotation. The amino nitrogen as determined by formol titration in mixtures of pneumococcus extract and serum (normal and immune) is practically the same before the toxic stage is reached and after it has disappeared. In other words, the polariscope here seems to be a better means of determining proteolysis than formol titration. The toxic substances here also seem to be intermediate products in the digestion of pneumococcus protein just as in the autolysates in NaCl solution. Its appearance and disappearance go hand in hand with evidences of proteolysis.

The action of normal guinea-pig serum on pneumococcus extracts has been studied further. The addition of serum to pneumococcus extracts before they have become toxic hastens the production of the toxic substance. Its addition during the height of toxicity may cause a diminution of the toxicity for a longer or shorter time, as is the case in the experiment given in Table 2. Serum added for the first time after the toxicity has disappeared by autolysis in NaCl solution rapidly restores the toxicity but not when added a second time. The action of sensitized guinea-pig serum on pneumococcus extracts is very similar except that its action is more rapid and often seems to yield more toxic material than normal serum.

From these observations the rapid parenteral digestion of pneumococcus proteins into identical toxic products in the sensitized animal on reinjection can scarcely be questioned. This conception would seem to afford the best explanation of the peculiar difference in the behavior of normal and sensitized pigs toward pneumococcus extracts and autolysates.

Dick[1] has shown that the proteolytic power of the serum toward pneumococcus protein is increased at the time of crisis in lobar pneumonia. This fact, together with the demonstration of the appearance and rapid disappearance of toxicity in immune serum, where there is greatest proteolysis, makes it seem likely that crisis

[1] *Jour. Infect. Dis.*, 1912, 10, p. 383.

in lobar pneumonia may be due in part to an increased proteolytic power of the serum on pneumococci.

MECHANISMS OF INTOXICATION WITH PNEUMOCOCCUS AUTOLYSATES AND OF INTOXICATION IN ANAPHYLAXIS COMPARED.

In order to determine further whether similar toxic substances are produced *in vivo* in immediate anaphylaxis as *in vitro* by autolysis of pneumococci, a series of experiments on normal guinea-pigs was made similar to those made by Auer and Lewis on sensitized guinea-pigs.

Briefly stated, the results obtained show that guinea-pigs which are deeply under the influence of ether, of chloroform, of urethan, or of morphin have much less or no bronchial spasm even when approximately one and one-half times the minimum but regularly fatal dose is injected. The anesthetics do not prevent death, however. Death here usually occurs in from 30 minutes to two hours. Some dyspnea is usually observed, then breathing becomes shallow, the animal is prostrated, and the temperature and leukocytes drop markedly. Pulmonary and more particularly cardiac, intestinal, and adrenal hemorrhages are often marked. Atropin in proper doses prevents bronchial spasm, just as Auer and Lewis[1] found in anaphylaxis, and as Mita[2] recently found in acute beef serum intoxication.

When 0.08 mg. of atropin sulfate is injected intravenously just before the extract, it prevents bronchial spasm completely even when one-third more than the regularly fatal dose of the extract is given; but when the same dose of atropin is injected immediately after the extract it fails to protect. The protective power if injected 15 minutes earlier is very much less than when injected immediately before the extract. The animals which are thus protected against the immediate death usually die over night. A comparison of the effect of ether and atropin when the same toxic extract is injected shows that the amount of hemorrhage is greater in the animals receiving ether than in the animals receiving atropin. The former die inside of two hours and the latter in 18 to 24 hours.

[1] *Jour. Exp. Med.*, 1910, 12, p. 151.

[2] *Ztschr. f. Immunitatsf.*, 1911, 11, p. 501.

It has been shown by Auer and Lewis also that the bronchial spasm in immediate anaphylaxis is due to peripheral action. If it could be shown that toxic pneumococcus autolysates have a similar peripheral action it would be evidence that the toxic substances in the two instances are similar. Accordingly the effect of a highly toxic pneumococcus autolysate was tested upon pithed normal guinea-pigs. The animals were partially anesthetized with ether, a glass canula was tied in the trachea, the vagi cut, and after artificial respiration was begun, the medulla, the lower brain, and the cord were destroyed by pithing. The blast of air, which was constant in volume and rate throughout the experiment, produced a well defined expansion of the chest. Enough time was allowed to elapse so that the effect of the ether had worn off. The doses selected were one and one-half times and twice the minimum fatal dose for untreated pigs of the same size (250 gms.). The injection (12 c.c.) was made into the jugular vein. Two to four minutes after the injection the chest expansion grew less and less, the chest became fuller until no oscillations could be made out between the blasts of air driven into the trachea. On opening the thorax the heart was found beating and the lungs distended and pale. A good sized slit (0.5 cm.) in the margin of the lung showed no escape of air with forced injections, and no bleeding. Pressure on the excised lung failed to collapse it and no bubbles of air escaped from the trachea when pressure was made under water, presenting an exactly similar picture to the lung in the unpithed animal and in the sensitized animal on reinjection. The air is imprisoned in the lung.

Adrenalin, 0.2 to 0.1 mg., when injected intravenously just before, together with, and immediately after toxic pneumococcus autolysate, prevents death from asphyxia by relieving the bronchial spasm, the animals usually dying soon after with marked pulmonary and other hemorrhages. The action of adrenalin is similar to that found by Jonuschke and Pollak[1] in cats following muscarin injection, and by Pal[2] in guinea-pigs following peptone injections.

Biedl and Kraus[3] were unable to protect anaphylactic guinea-pigs and normal guinea-pigs injected with peptone by forced

[1] *Arch. f. Exp. Path. u. Pharm.*, 1911, 66, p. 205.

[2] *Deutsche med. Wchnschr.*, 1912, 38, p. 5.

[3] *Ibid.*, 1911, 37, p. 1300.

tracheal respiration, whereas they were able to protect against acute death following injection of "anaphylatoxin," and hence they draw the conclusion that the mechanism of the latter must be different from that of peptone. I have made a similar study of the effect of forced tracheal respiration in normal and sensitized guinea-pigs. The results briefly stated are these: Forced respiration protects both normal and sensitized guinea-pigs against approximately one-fourth more than the minimum fatal dose of toxic pneumococcus autolysate in the normal, and of unautolyzed pneumococcus extracts in the sensitized animals. But against larger doses it fails to protect in each case. Hence it is clear that the protective power of this procedure is in both cases a matter of degree.

Finally, repeated tests show conclusively that injections into the left heart of toxic pneumococcus autolysates require a considerably larger dose to kill normal guinea-pigs regularly than injections into the jugular vein or right heart, whereas in sensitized animals the reverse is true. This fact is in harmony with the idea that there occurs a rapid digestion of the unautolyzed extract into toxic material in the sensitized animal, injection into the left heart affording greater opportunity for enough material to be formed to produce bronchial spasm.

In this connection another point which has been observed should be mentioned. While it requires a larger dose of pneumococcus autolysates to produce fatal bronchial spasm on left heart than on jugular injection the systemic spasms are more pronounced.

The symptoms produced, the condition of the lungs after death, and the facts brought out here show clearly that death following injections of toxic pneumococcus autolysates is due to asphyxia from bronchial spasm, as in anaphylaxis. The results speak strongly in favor of the view that similar toxic products are concerned in each case.

In this connection it should be stated that while the serum of guinea-pigs dying in immediate anaphylaxis (horse serum) has been found to produce the milder symptoms of anaphylaxis like those following non-fatal injections of toxic pneumococcus autoly-

sates—dyspnea, irritability, drop in temperature and leukocytes—it has not been possible to produce fatal bronchial spasm.

RESTORATION OF TOXICITY OF AUTOLYZED PNEUMOCOCCUS EXTRACTS.

Extract 258, used in the experiments in Table 3, was prepared in the usual way by suspending pneumococci in NaCl solution at 37° C. and testing the toxicity at intervals by injecting 7 c.c. into the jugular vein. It produced no immediate symptoms when first made, definite symptoms at the end of 24 hours, death in 4 minutes at the end of 48 hours, and again no symptoms at the end of 96 hours. The experiments were made after this extract had been kept in the ice-chest for 6 months; 8 c.c., and 6 c.c. to which 2 c.c. of NaCl solution were added, now produced no symptoms. It is

TABLE 3.

RESTORATION OF TOXICITY OF PNEUMOCOCCUS AUTOLYSATES.

Mixtures	Symptoms*
Extract 258, 6 c.c.+normal guinea-pig serum, 2 c.c. at once	No symptoms
Extract 258, 6 c.c.+normal serum, 2 c.c. after 1 hour at 37° C.	Death in 3 minutes
Extract 258, 6 c.c.+normal serum after 2 hours at 37° C., then in ice-chest, 48 hours	No symptoms
Extract 258, 6 c.c.+normal serum at 37° C. 2 hours and 1 hour after adding fresh serum a second time†	No symptoms
Extract 258, 6 c.c.+normal serum 2 c.c. heated (60° C. one-half hour) 37° C. 1 hour	Definite symptoms
Extract 258, 6 c.c.+2 c.c. serum+charcoal 1 gm. 1 hour at 37° C.	Slight symptoms
Extract 258, 6 c.c.+2 c.c. normal serum treated with charcoal (10 gms. per c.c. serum at 37° C. 1 hour), then at 37° C. 1 hour	No symptoms
Extract 258, 6 c.c.+charcoal (1 gm. per c.c. at 37° C. 1 hour)+2 c.c. normal serum, then at 37° C. 1 hour	No symptoms
Extract 258, 6 c.c.+2 c.c. normal serum which has been extracted with ether, then at 37° C. 1 hour	No symptoms
Extract 258, 6 c.c.+2 c.c. ether extracted serum to which the equivalent amount of ether soluble substance is again added, at 37° C. 1 hour	No symptoms

* Control experiments, in which corresponding mixtures of serum and NaCl solution were injected, failed to give symptoms.

† A closer study of the effect of adding serum a second time to reactivated extracts by injecting the mixtures at various intervals shows that the serum now fails entirely to cause a return of toxicity.

shown in Table 3 that normal guinea-pig serum reactivated temporarily the toxicity of pneumococcus autolysates which had lost their toxicity. The second addition of serum after the toxicity has disappeared no longer has this power. It would seem as if there had occurred a certain equilibrium in the autolysate so that the autolytic ferment would no longer convert into toxic material fractions of the pneumococcus protein which, however, are acted on under the new conditions. The active principle which makes new

toxic material in normal serum seems to be complement, because it is theromolabile, adsorbed by animal charcoal, and removed and destroyed by ether. The material from which normal serum makes this toxic substance is also removed by animal charcoal. That mixtures of heated serum and pneumococcus extract become toxic has been observed and deserves further study because it suggests that here toxic material may be produced by proteolytic action of the extract on the heated serum.

The observation that a leukocytic pneumococcus extract which has become non-toxic becomes toxic in 4 hours on the addition of new pneumococci is in favor of the view that the proteolytic action of these pneumococcus leukocytic extracts play a definite rôle in the production of toxic substances. Moreover, if pneumococcus autolysates which have become non-toxic are heated, it takes longer for normal serum to render them toxic again, and at times it fails entirely to do so.

TABLE 4.

Restoration of Non-toxic Leukocytic Extracts of Pneumonic Lungs by Normal Serum.

The Mixtures Were Kept at 37° C. 7 Hours and on Ice 20 Hours before Injection	Symptoms in Normal Guinea-Pigs Following Jugular Injection of 7 c.c.
Extract "A,"* 4 c.c.+normal guinea-pig serum, 2 c.c.	Death in 4 minutes
Extract "A," 4 c.c. heated 60° C. 1 hour+normal guinea-pig serum, 3 c.c.	Very slight symptoms
Extract "A," 4 c.c.+heated (60° C. 1 hour) normal guinea-pig serum, 3 c.c.	Definite but mild symptoms
Extract "A," unheated 3 c.c., heated 1 c.c.+normal guinea-pig serum, 3 c.c.	Severe symptoms but recovered

* Extract "A" was prepared as indicated below in Table 5, after being kept at 37° C. for 3 days.

VARIATIONS IN THE SUSCEPTIBILITY OF GUINEA-PIGS TO TOXIC PNEUMOCOCCUS AUTOLYSATES.

Jugular injections.—Guinea-pigs vary considerably in their susceptibility to the toxic action of pneumococcus autolysates. This is especially true of those which have been fed carrots. The difference in susceptibility is much less marked in guinea-pigs which are starved, and the general susceptibility is greater. It has been found that the minimum fatal dose for guinea-pigs which have been starved for 60 hours is approximately one-third less than that required for well fed pigs of the same weight. For these reasons it

has been the rule not to feed the animals greens, especially carrots, on the day of the experiments. Other food, such as oats and hay, makes less difference.

The study which has been made of the altered susceptibility of guinea-pigs following various injections may be summarized as follows: Filtrates of broth cultures of pneumococci, autolyzed extracts, both before and especially after the toxic stage, and to a lesser degree autolyzed pneumococci, render guinea-pigs insusceptible to doses of pneumococcus autolysates, otherwise fatal, in 2, 4, 6, 24, and 48 hours. The animals do not have bronchial spasm and remain permanently well. The control injections of broth, NaCl solution, and of extracts of typhoid bacilli do not render guinea-pigs insusceptible to pneumococcus autolysates.

Portal injections.—The power of the liver to neutralize various poisons is well known. The close relation of the liver to infection in general and more particularly to bacterial intoxications, which have their origin in the intestinal tract, as well as the fact that typhoid bacilli and colon bacilli have been found in my experiments to yield on autolysis an exactly similar toxic material to that yielded by pneumococci, seems to indicate that the effect of portal injections of highly toxic autolysates should be studied.

Experiments were made under ether and urethan anesthesia by injecting the material into the main branch of the portal vein, but the results were so unsatisfactory that a different method was used. This consisted in making a small incision in the abdomen from 0.5 to 1 cm. in length, and injecting the toxic dose into a small branch of the mesenteric vein of the small intestines. The needle employed was of the same caliber as the one for jugular injections. The results obtained are striking. Well fed guinea-pigs fail to develop the acute symptoms even when one and one-half times the minimum fatal dose by the jugular is injected. The animals are made only slightly ill. Starved guinea-pigs (48 to 60 hours), on the other hand, react almost as promptly as on jugular injection, and with typical symptoms. Two hours after they have eaten ravenously of carrots, the acute symptoms are again very much reduced, and in 24 hours after feeding they again behave as normal pigs.

Complement titration and opsonic determination of the serum from normal pigs and from starved pigs fail to show a measurable difference in complement content and in opsonins for pneumococci. The late toxic action in the starved animals was also strikingly greater than in those well fed, although here the liver was not always sufficient to protect the well animals against a fatal outcome.

In other words, the protective power of the liver against toxic bacterial products may be related in some way to its glycogenic function. It is possible, however, on the other hand, that this mechanism is related to the digestive function. These experiments would seem to explain why great fatigue and hunger render the human, especially children, more susceptible to intoxication, particularly of intestinal origin. It is clear that the behavior of the starved animals toward toxic pneumococcus autolysates does not depend on the complement content of the serum.

Because fresh normal serum has the power to restore toxicity to autolysates which have lost it, the effect of extreme hemorrhage on the susceptibility of otherwise normal pigs was also studied. Four normal guinea-pigs were injected with a known toxic autolysate after having been bled varying amounts by cutting the carotid artery. Two were injected intraperitoneally with NaCl solution, and were bled until the hemoglobin was 20 per cent. The other two were bled nearly to death. All four animals died from typical symptoms in the usual way, the symptoms if anything being more marked than usual. Hence the conclusion seems warranted that the toxic substance exists in the autolysate, and is not made on injection by the action of complement, and that guinea-pigs are more susceptible to the action of the substance after acute hemorrhage.

OBSERVATIONS ON THE TOXICITY OF PNEUMOCOCCUS EXUDATES, ETC.

The question whether or not toxic material is produced *in vivo* in pneumococcus infections similar to that *in vitro* on autolysis of pneumococci, and whether it disappears under similar conditions, was taken up also. A study of the blood of guinea-pigs dying from pneumococcemia, of the peritoneal exudate in guinea-pigs with pneumococcus peritonitis, of pus in pneumococcal empyema, and

of consolidated pneumonic lungs brings out the fact that in all these cases there is produced similar toxic material. Thus the serum of guinea-pigs dying from pneumococcemia, while it has never been found to contain enough free toxic material to produce bronchial spasm in normal guinea-pigs when first obtained, may produce fatal shock in normal pigs in 4 c.c. doses when kept at 37° C. for from 6 to 24 hours, and fatal immediate anaphylaxis in sensitized pigs as soon as drawn.

A portion of a consolidated lung in gray hepatization, still quite warm, which contained very many pneumococci, some well preserved and many in various stages of disintegration, was ground up with a meat chopper, strained through a thick layer of gauze after adding enough NaCl solution so that the strained material was of a thick syrupy consistence. This suspension was grayish in color, and was diluted with eight times its volume of NaCl solution, thoroughly shaken, and injected at once into the jugular vein of a normal and a sensitized pig of the same weight. The normal pig died in 5 minutes, the sensitized in 3 minutes, of typical symptoms. Ether was added to one portion of this suspension, which was then placed at 37° C. for three days. Injection of this material into two normal pigs was not followed by acute symptoms. Another portion, 250 c.c., was washed in NaCl solution resuspended in NaCl solution, and divided into two equal parts (A) and (B). To (A) virulent pneumococci from 300 c.c. of broth culture were added. To both parts 10 c.c. of ether were added and allowed to evaporate through the cotton plugs. The toxicity of these mixtures was then tested as indicated in Table 5. Cultures on blood agar made at the time of the injection proved sterile.

From the tests with the unwashed and washed lung extracts it is clear that there is produced in pneumonic lungs a highly toxic substance. That the pneumococcus plays an essential rôle in the formation of this acutely toxic substance is shown by the fact that when new pneumococci are added [(B) Table 5] the toxicity for both normal and sensitized pigs disappears later than when no new pneumococci are added [(A) Table 5]. Moreover, these extracts, consisting, as they do, chiefly of leukocytes and pneumococci, rapidly make toxic material from new pneu-

mococci after their toxicity has disappeared, as indicated in the last two experiments.

From these results we see that the same peculiar difference in the behavior of normal and sensitized pigs exists toward extracts of pneumonic lungs as to pneumococcus extracts in NaCl solution.

TABLE 5.

TOXICITY OF SUSPENSIONS OF PNEUMONIC LUNG MIXED WITH VIRULENT PNEUMOCOCCI.

Mixtures	Appearance of Pneumococci	Symptoms in Guinea-Pigs (7 c.c. into Jugular Vein)	
		Normal	Sensitized
(A) After 30 minutes at room temperature	Mostly gram positive Some gram negative	No symptoms	Death in 6 minutes
(B) After 30 minutes at room temperature	Mostly gram positive Some gram negative	Severe symptoms	Death in 3 minutes
(A) After 20 hours at 37° C.	Many gram negative	Severe symptoms. Nearly dead in 3 minutes	No symptoms
(B) After 20 hours at 37° C.	Many gram negative	Death in 3 minutes	Very severe symptoms. Nearly dead in 10 minutes
(A) After 48 hours at 37° C.	Nearly all gram negative	Slight symptoms	No symptoms
(B) After 48 hours at 37° C.	Nearly all gram negative	Severe symptoms. Nearly dead in 10 minutes	Slight symptoms
(A) After 72 hours at 37° C.	Nearly all gram negative	No symptoms	
(B) After 72 hours at 37° C.	Nearly all gram negative	Slight symptoms	No symptoms
(B) After 96 hours at 37° C.	Nearly all gram negative	No symptoms	No symptoms
(A) After 1 hour at 37° C. +unautolyzed vir. pn. from 6 c.c. broth at 37° C. for 4 hours	Both gram negative and gram positive	Severe symptoms, including bronchial spasm, but recovered	
7 c.c. NaCl sol. unautolyzed vir. pn. from 6 c.c. broth at 37° C. 4 hours	Nearly all gram positive	No symptoms	

The appearance and disappearance of the toxicity, however, occur earlier in the leukocytic pneumococcus mixtures. Similar results have been obtained in dogs.

A similar study of extracts in NaCl solution of consolidated lungs (for which I am indebted to Dr. Le Count) shows that the amount of toxic substance which kills guinea-pigs by provoking bronchial spasm, while present at all stages of consolidation, is greatest in those which contain mucoid exudate and from which smears and cultures show the presence of the greatest number of pneumococci as well as the greatest evidence of disintegration of pneumococci and leukocytes.

Control experiments in which extracts in NaCl solution of normal lungs were prepared show that the amount of lung tissue must be larger and that the symptoms produced are not typical, death occurring somewhat later than when extracts of pneumonic lungs were used. This result is in harmony with the results of Dold[1] who found that extracts of various organs may be toxic on intravenous injection.

SUMMARY.

The toxic substances obtainable from pneumococci on autolysis in NaCl solution, from pneumococcus-leukocyte mixtures, from pneumococcus exudates, from the action of normal and immune serum on pneumococci, produce identical symptoms in normal guinea-pigs, and these symptoms are indistinguishable from those of immediate anaphylaxis. Postmortem appearances are also similar. The serum of sensitized guinea-pigs produces the toxic material from pneumococcus extracts more rapidly than normal serum and probably also in larger amounts. This is associated with a more rapid proteolysis as measured by the polariscope.

Morphin, ether, urethan, atropin, and adrenalin protect normal guinea-pigs against the toxic material, obtained *in vitro* from pneumococci, and also sensitized guinea-pigs on reinjection.

Forced respiration fails to protect in each instance when the doses are properly gauged.

The action of the toxic substances on normal guinea-pigs is peripheral, as in immediate anaphylaxis.

The behavior of normal and sensitized guinea-pigs toward unautolyzed extracts of pneumococci, which are non-toxic to the former and very toxic to the latter, toward partially autolyzed extracts, which are very toxic to the former, and slightly or not at all to the latter, and toward more completely autolyzed extracts, which are non-toxic to both, speaks strongly in favor of the view of a rapid parenteral digestion into toxic cleavage products in sensitized animals.

The serum obtained from guinea-pigs in anaphylactic shock has been found to contain substances which produce symptoms

[1] *Ztschr. f. Immunitätsf.*, 1911, 10, p. 53.

in normal pigs indistinguishable from the milder symptoms of anaphylaxis and from those following injections of non-fatal doses of pneumococcus autolysates.

In view of these facts the conclusion seems warranted that the symptoms in anaphylaxis are due to toxic protein cleavage products, formed rapidly after reinjection, and identical with the toxic products obtained *in vitro*.

Normal guinea-pigs vary considerably in their susceptibility to jugular injections of toxic pneumococcus autolysates just as they do to intoxication on reinjection of proteins. When starved they are more susceptible and the individual variations are less marked. Well fed guinea-pigs are quite insusceptible to portal injections, while those starved are nearly as susceptible to portal injections as to jugular injections.

After pneumococcus autolysates have become non-toxic, their toxic power may be restored by the addition of normal or immune serum, by leukocytes, by the addition of unautolyzed pneumococci, and by extracts. The addition of serum or leukocytes a second time, however, fails to restore the toxicity unless new extract is added.

The appearance and disappearance of the toxic substances seem to be definitely related to proteolysis. This is true alike in pneumococcus autolysates, in serum mixtures, in leukocytic mixtures, and in pneumococcus exudates.

A single intravenous injection of non-fatal doses of extracts before they have become toxic, while highly toxic, and especially after the toxic stage is passed, or of autolyzed pneumococci, renders guinea-pigs insusceptible to subsequent injections of toxic pneumococcus autolysates.

Recovery from pneumococcus infections, the crisis in lobar pneumonia, for example, probably occurs when the toxic substances of the pneumococci have been digested beyond the toxic stage. The factors which would seem to be concerned in this process are the autolytic ferment of the pneumococcus, the increased proteolytic power of the serum, the proteolytic action of the leukocytes, and the increased opsonic power of the serum, with a consequent greater phagocytosis.

STUDIES ON ENDO'S MEDIUM, WITH OBSERVATIONS ON THE DIFFERENTIATION OF BACILLI OF THE PARATYPHOID GROUP.*

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Of the many media at present in use for the routine isolation of typhoid bacilli from stools and urine, the medium of Endo is probably the one most widely employed. While this medium is easy to apply and reliable, its production is fraught with a certain amount of difficulty, and irregularities in the character of the finished medium often result.

Kastle and Elvove[1] have pointed out that the chief cause of irregularity is the varying purity of sodium sulfite as commercially obtainable. They overcame this difficulty by using anhydrous sodium sulfite instead of the crystallized variety which is hydrated.

The work here recorded was begun with the purpose of determining the chemical principles on which depend the color reactions taking place in this medium in the hope that, after fully understanding these, changes might be introduced which would lead to simplification of production, and greater stability of the end product.

Endo, in his original article,[2] said: "Der Säurekomponent des roten Rosanilin Salzes wird durch Reductionsmittel, wie Na_2SO_3, leicht reduziert. Das dadurch entfärbte Rosanilin verbindet sich mit der durch Coli Bacterium produzierten Säure, und der Nährboden färbt sich schön rot." This statement, while offering a plausible explanation, which seems to have been accepted, was apparently not based on actual experimental investigation.

The colorless solution obtained by treating fuchsin with Na_2SO_3,

* Received for publication May 15, 1912.

[1] *Jour. Infect. Dis.*, 1909, 6, p. 619.

[2] *Centralbl. f. Bakt.*, I, Orig., 1904, 35, p. 109.

$NaHSO_3$, or SO_2 in solution is used extensively in chemical analysis for the detection and even colorimetric quantitative determinations under the name of "Schiff's aldehyde reagent."[1] Acids do not give any color test with this reagent and, if present in sufficient quantity, prevent aldehydes from giving it. These well known facts contradict Endo's statement. The reaction taking place between fuchsin, Na_2SO_3, and aldehydes as given by Nietski[2] is as follows: "Rosanilin and pararosanilin form with sulfurous acid and the alkali bisulfites colorless, easily decomposed compounds. By the action of aldehydes upon these bodies peculiar violet dyes are formed."

This explains why the medium is red when hot, and colorless when cold, since the compound upon dissociating would liberate the free fuchsin.

It is reasonable to suppose that the same reaction takes place in Endo's medium since it is to be expected that aldehydes are intermediate products in the oxidation of carbohydrates to acids by bacteria.

Preliminary experiments were made to determine whether the reaction of various bacteria on a medium made up like that of Endo, except that various carbohydrates were substituted for lactose, would correspond to the production of acid by these bacteria from the same carbohydrates.

TABLE I.

MEDIUM MADE UP WITH SODIUM SULFITE AND FUCHSIN AS IN ENDO'S MEDIUM, CONTAINING VARIOUS CARBOHYDRATES.

	Dextrose	Mannite	Maltose	Lactose	Sucrose	Dextrin
Fecalis alkaligenes	Pink	Pink	Pink	Pink	Pink	Pink
B. dysenteriae, Shiga-Kruse	Red	White	White	White	White	White
" " , Hiss-Russell	"	Red	"	"	"	"
" " , Flexner	"	"	Red	"	Red	Light Red
B. typhosus	"	"	"	"	White	"
B. schottmüller-müller	"	"	"	"	"	White
B. coli communis	"	"	"	Red	"	"
B. coli communior	"	"	"	"	Red	Red

Compare with:

[1] S. P. Mulliken, *Identification of Pure Organic Compounds*, 1904, 1, p. 15.

[2] *Chemie der Organischen Farbstoffe*, 1906, p. 163; Victor Meyer, *Berichte*, 13, p. 2343.

ACID FORMATION.

(From Hiss and Zinsser, *A Textbook of Bacteriology*, p. 443.)

	Dextrose	Mannite	Maltose	Lactose	Sucrose	Dextrin
Fecalis alkaligenes............	......	......	......	......	......	......
B. dysenteriae, Shiga-Kruse....	Acid	......	......	......	......	......
" " , Hiss-Russell....	"	Acid	......	......	......	......
" " , Flexner........	"	"	Acid	......	Acid	Acid
B. typhosus..................	"	"	"	......	......	"
B. schottmüller-müller.........	"	"	"	......	......	......
B. coli communis.............	"	"	"	Acid	......	......
B. coli communior............	"	"	"	"	Acid	Acid

It is evident that color production on fuchsin-sulfite agar follows the acid chart with the one exception that *B. fecalis alkaligenes* in all cases gave pink colonies. This is in direct agreement with the fact that alkalis react with "Schiff's reagent" to give a pink color.[1]

After the second or third day the plates became pink throughout, as is usual with Endo's medium when exposed to the light and air. In from five to 10 days later, when the red colonies had attained considerable size, they began to decolorize the medium surrounding them, the body of the colony remaining red owing to the fact that the bacteria had taken up the stain. We account for this in the following manner: The aldehyde, as fast as produced, combines with the reagent and is fixed, thus being prevented from oxidizing to acid. But upon continued growth of the colony, all of the fuchsin sulfite in the immediate neighborhood is used up. As soon as this occurs, the aldehyde, still being formed, is oxidized to acid and this acid decolorizes the medium. A part of the decolorized medium was tested with litmus and the leukobase of "soluble blue"[2] and showed an acid reaction. By placing a drop of dilute acid on that portion of a plate which had been colored by the growth of the bacteria, we produced a similar effect. A drop of dilute alkali, on the other hand, intensified the color.

Owing to the fact that Na_2SO_3 is very easily oxidized and that most samples therefore vary in SO_2 content, we prepared our fuchsin sulfite reagent by adding a solution of the sulfite to a

[1] *Loc. cit.*

[2] "Soluble blue," the commercial term for an anilin blue soluble in water and easily reduced to its leukobase by treatment with ammonia and zinc dust.

measured quantity (5 c.c. saturated alcohol solution per liter) of fuchsin until we obtained the maximum delicacy of reaction with a dilute solution of formaldehyde. This solution of the reagent was then poured into the hot 3 per cent lactose agar. The medium thus prepared gave us the best color reaction with the bacteria. This we take as an additional proof that the color is due to aldehyde formation. To keep the color from spreading, however, it was found necessary to add a slight excess of sulfite.

The differentiation of typhoid and colon bacilli on Endo's medium on the basis of aldehyde formation is, as far as we can ascertain, the only application of this principle to the separation of bacterial species. It seemed to the writers that this principle might serve to base differentiation on aldehyde formation from the various carbohydrates in the same way that such differentiations are now made on the basis of acid formation. For this reason tests were made with other members of the colon-typhoid-dysentery group as follows:

TABLE 2.

MEDIUM MADE UP OF FUCHSIN-SULFITE AGAR AS IN ENDO'S MEDIUM—WITH ADDITION OF CARBOHYDRATES AS FOLLOWS:

	Inulin	Dulcite	Rhamnose	Raffinose	Arabinose	Xylose	Glycogen
Fecalis alkaligenes..	Pink	Pink	Pink	Pink	Pink	Pink	No growth
B. dysenteriae, Shiga-Kruse.....	White	White	White	White	White	White	"
B. dysenteriae, Hiss-Russell.....	"	"	"	"	"	"	"
B. dysenteriae, Flexner.........	"	"	"	"	Late Red	"	"
B. typhosus........	"	"	"	"	White	Red	"
B. schottmüller-müller..........	"	Late Red	"	"	Red	"	"
B. coli communis...	"	Red	Red	"	"	"	"
B. coli communior..	Red	"	"	Red	"	"	"

The colors produced on inulin, dulcite, and rhamnose were rather weak and indefinite, and therefore of no differential value. Raffinose differed from sucrose only in its behavior toward the dysentery bacillus of the Flexner type. In the glycogen medium no increase in growth took place and after two days the bacteria were evidently all dead, since we could obtain no growth upon agar slants on transplantation. But when cultures were planted upon media containing the same amount (10 gms. per liter) of

glycogen but no fuchsin sulfite, they grew excellently, showing that the bactericidal effect was not due to the glycogen itself.

The reactions with the xylose medium appeared to us particularly interesting in that they showed differences between *B. typhosus* and *B. dysenteriae*, the former giving colored, the latter white colonies.

Arabinose in the same way seemed to offer a means of differentiating *B. typhosus* from the paratyphoid group.

Wishing to determine whether this was true for all of the members of the paratyphoid group we made a more thorough study of the behavior of the paratyphoid bacilli which were available at that time in this laboratory. The arabinose and xylose media together divided our cultures into three groups, viz.: (1) those that gave color with both; (2) those that gave color with arabinose and not with xylose; (3) those that gave color with xylose and not arabinose.

Later we were able to repeat these tests with a larger number of cultures of the intermediate group from various laboratories.[1] The cultures we obtained in addition to our own and their source were as follows:

B. paratyphosus "A" No. 25,	University of California
B. paratyphosus "B" No. 62,	" " "
B. libman,	Mt. Sinai Hospital, New York
B. seeman,	" " " " "
B. gärtner,	" " " " "
B. paratyphosus No. 7,	Loomis Laboratory New York
B. paratyphosus No. 116,	" " " "
B. paratyphosus schottmüller,	" " " "
B. enteritidis No. 18,	" " " "
B. enteritidis No. 69–5,	" " " "
B. enteritidis No. 61–9,	" " " "
B. enteritidis No. 132,	" " " "

All the cultures used in our experiments were plated and fished to avoid errors of contamination. They were all gram negative bacilli of characteristic appearance, motile, and reacted as follows on the media mentioned:

[1] We take pleasure in expressing our gratitude to Professor Ravenel of Wisconsin, Professors Winslow, Libman, Torrey of New York, and Professor Fitzgerald of Berkeley for cultures kindly sent to us.

Gelatin—no liquefaction.
Hiss tube medium—motility and gas.
Dextrose broth—gas.
Lactose broth—no gas; no acid.
Sucrose broth—no gas; no acid.

Our results on xylose and arabinose fuchsin-sulfite agar were as follows:

TABLE 3.

COLOR REACTION OF MEMBERS OF THE SO-CALLED PARATYPHOID GROUP OF BACILLI ON FUCHSIN-SULFITE AGAR CONTAINING ARABINOSE AND XYLOSE RESPECTIVELY:

	Arabinose	Xylose
Group 1, Color Reaction on Both Sugars		
B. schottmüller-müller (Columbia University)	Red	Red
B. schottmüller-seeman (Columbia University)	"	"
B. schottmüller (Loomis)	"	"
B. seeman (Mt. Sinai)	"	"
B. enteritidis (Am. Museum Nat. Hist.)	"	"
B. enteritidis "No. 18" (Loomis)	"	"
B. enteritidis "No. 69–5" (Loomis)	"	"
B. enteritidis "No. 61–9" (Loomis)	"	"
B. enteritidis "No. 132" (Loomis)	"	"
B. gärtner (Enteritidis) (Mt. Sinai)	"	"
B. paratyphosus "B" (University of Chicago)	"	"
Group 2, Color on Arabinose but Not on Xylose		
B. typhi murium (Columbia University)	"	White
B. paracolon gwynn (Columbia University)	"	"
B. paratyphosus "A" (University of Chicago)	"	"
B. paratyphosus "No. 7" (Loomis)	"	"
B. paratyphosus "No. 116" (Loomis)	"	"
B. libman (Mt. Sinai)	"	"
Group 3, Color on Xylose but Not on Arabinose		
B. hog cholera (University of Wisconsin)	White	Red

Twenty-five strains from the colon-typhoid-dysentery group were tried out on xylose and arabinose broth to see whether aldehyde and acid formation always occurred together. This was found to be true for all the strains we tried. The indicator we used for showing acid formation was the leukobase of "soluble blue." The use of this compound as an indicator for the fatty acids and our results concerning the identification of *B. typhosus* by the use of xylose broth, in which it is the only member of the whole group which ferments the sugar without gas formation, will be discussed in our next communication.

SUMMARY AND CONCLUSIONS.

It is seen from our results that by the use of the sugars xylose and arabinose in media, the bacilli of the so-called paratyphoid or intermediate group may be divided into definite subgroups.

We regret that we had only 18 strains with which to work but believe that the definite and constant variations observed should serve to indicate a method by which the organisms of this group, now so difficult to classify, may be eventually differentiated. We believe that these differentiations as well as others, when obtained on the fuchsin-sulfite media of Endo, depend not on acid formation but on aldehyde production from the carbohydrates.[1] Indeed a differentiation may be possibly further obtained when strong acid formation occurs, by subsequent decolorization of the medium surrounding the individual colonies.

When arabinose and xylose are used, however, differentiations within this group may be obtained with a simple acid indicator.

[1] H. Kahlenberg, *Ueber die Bildung und Vergärung von Ameisensäure durch Bacterium Coli Commune*. Heidelberg, 1911, p. 14.

A CRITICAL STUDY OF THE ORGANISMS CULTIVATED FROM THE LESIONS OF HUMAN LEPROSY, WITH A CONSIDERATION OF THEIR ETIOLOGICAL SIGNIFICANCE.*†

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CONTENTS.

INTRODUCTION.

In a recent communication the writers[1] announced that more than one strain of acid-fast bacilli could be cultivated by special methods from the lesion of human leprosy, and drew special attention to a non-chromogenic form which cannot be made to adapt itself to a vegetative habit and even though many generations removed from the parent stem will not grow on ordinary media, nor, indeed, on any but specially prepared nutrients containing broken-down protein.

* Received for publication May 17, 1912.

† Read before the American Dermatological Association, St. Louis, May 23, 1912.

[1] *Jour. Am. Med. Ass.*, 1912, 58, p. 1427.

A preliminary study of the different strains of organisms encountered in the course of our investigations of leprosy from the standpoint of its bacteriology convinced us that further research on the types of organisms cultivated was necessary, and the present paper is the result of a comparative consideration of the various strains isolated from leprous patients by different workers.

It is our intention to continue our investigations on this subject until we have attempted to settle finally the various mooted points mentioned in the following paragraphs.

OCCASION AND SCOPE OF THE PRESENT PAPER.

In the course of our work we have isolated and grown from eight different cases of leprosy an acid-fast bacillus which is non-chromogenic and cannot be cultivated in our hands except in the presence of an amino-acid medium. This strain, which was described somewhat in detail by one of us (Duval[1]) in 1910, is entirely unlike the chromogenic leprosy cultures subsequently isolated by ourselves and others.

A review of the literature, together with a careful study of our cultures and preparations, has convinced us that two and possibly three apparently different organisms have been cultivated from the specific lesions of leprosy, namely: (1) a non-acid-fast diphtheroid (Kedrowski), (2) an acid-fast chromogenic bacillus (Clegg), and (3) a permanently acid-fast bacillus (Duval) which *in vitro* maintains, well within specific limits as accepted, for instance, for *B. tuberculosis*, the morphology of the Hansen bacillus of the tissues and grows under artificial conditions only in the presence of special nutrients.

Whether the three varieties described by the above authors represent the same or distinct species, some one of which is the real exciter of leprosy and the others simply extraneous or accidental commensals, is a problem which we have attempted to solve by a comparative study of the lesions induced experimentally, by the behavior of the cultures with respect to immune sera, and by other well known methods. Furthermore we have gone back over some of the leprosy cases formerly studied to determine, if possible,

[1] *Jour. Exper. Med.*, 1910, 12, p. 649.

which yield the chromogenic and which the non-chromogenic strains above mentioned, or whether from any case the two types may be cultivated.

In other words, we have attempted to answer the following queries:

1. In what percentage of the lepers observed by us and at what stage and type of the disease does the chromogenic strain (Clegg), the non-chromogenic type (Duval), and the diphtheroid type (Kedrowski) exist in the lesions, and do any or all of the acid-fast strains grow outside of the animal body under conditions as non-acid-fast diphtheroids?

2. Are streptothrical forms cultivated from lepers in this region?

3. What are the relations between the chromogen of Clegg and the non-chromogen of Duval from the leprous lesion and what are the relations of both to other known acid-fast bacteria?

4. What is the value of animal experiments and serological tests in differentiating the bacteria isolated from leprous lesions?

5. Can an etiological rôle for any of the cultures studied by us be established, to the exclusion of the others, either by means of serum reactions or by a study of histo-pathological differences in the experimental lesion?

HISTORICAL.

Following upon the discovery by Hansen[1] in 1872 of an acid-fast bacillus in the leprous lesion to which he ascribed an etiological rôle, numerous investigators reported success upon the artificial cultivation of the specific organism of leprosy.

In general it may be stated that the earlier workers, among whom may be mentioned Bordoni-Uffreduzzi,[2] Babes,[3] Ducrey,[4] Czaplewski,[5] Spronck,[6] and others, isolated and described cultures which tinctorially and morphologically differed from the Hansen bacillus of the tissues, and though they claimed to have induced experimental lesions and to have fulfilled other postulates their results have not been universally accepted.

Kedrowski[7] in 1901 described an organism which he cultivated from the leprous lesion and believed to be the specific bacillus of leprosy. This author described his culture as a non-acid-fast diphtheroid bacillus which when injected into laboratory animals became acid-fast after a sojourn of weeks in the tissues. He advanced the theory that the acid-fast rods seen in human leprous lesions represent but a stage in the developmental cycle of a single pleomorphic species.

[1] *Norsk. Mag. f. Laegevidensk.*, 1874.

[2] *Ztschr. f. Hyg.*, 1884, 3, p. 178.

[3] *Ztschr. f. Hyg.*, 1889, 5, p. 173.

Giorno italiano dello Med. ver., 1892, 27, p. 76.

[5] *Centralbl. f. Bakt.*, 1897, 23, p. 97.

[6] *Semaine méd.*, 1898, 18, p. 393.

[7] *Ztschr. f. Hyg. u. Infectionskr.*, 1901, 37, p. 52

Deycke[1] and also Rost and Williams[2] have since reported upon the successful cultivation from the leprous nodule an organism similar to that of Kedrowski together with which they also found streptothrical forms and acid-fast rods.

More recently (1912) Bayon[3] describes a non-acid-fast diphtheroid obtained from a leper which behaves in a like manner to Kedrowski's, i.e., the initial growth from the tissues is non-acid-fast and a diphtheroid until passed through rats, after which it permanently changes into a typical acid-fast bacillus. Like Rost and Williams, he also mentions streptothrical forms in his culture. He concludes that not only is his culture identical with Kedrowski's but also that it is the cause of human leprosy, basing his argument upon specific reactions obtained with human leper serum and also upon the production of characteristic lesions in laboratory animals.

Clegg[4] in 1909 announced his success in the cultivation of an acid-fast bacillus which he isolated from lesions in a large series of lepers in the Philippines. He reported that multiplication in each instance occurred in the transferred leprous tissue bits when planted with amebae and their symbionts. He subsequently obtained pure cultures of the acid-fast organisms on the ordinary laboratory media as a moist profuse pigmented growth after heating at 60° C. for 30 minutes to kill out the symbionts.

Duval[5] (1910) was the first to confirm Clegg's work, and described a method by which the acid-fast bacilli in the leprous lesion could be cultivated *in vitro* without the use of symbionts. Duval's culture differed from Clegg's in that it did not produce pigment and refused to grow except upon special nutrients.

Acid-fast cultures similar in every respect to Clegg's have since been reported by Duval[6] in Louisiana, Brinkerhoff, and Currie[7] in Honolulu, Rivas[8] in Philadelphia, Thompson[9] in Australia, Wellman[10] in California, and by other workers in Hawaii.

CRITICAL NOTE.

We wish particularly to draw attention here to the curious results obtained by various workers with leprosy organisms who believe that they have in pure culture bacteria of such protean pleomorphism that these may alternately appear as non-acid-fast diphtheroids, as both acid-fast and non-acid-fast streptothrices, and as ordinary acid-fast rods. For instance, Bayon,[11] after detailing his somewhat disheartening experience with various methods of study, writes: "I now took in hand the non-acid-fast streptothrix and the acid-resisting diphtheroid I had cultivated from a leper at this school, injecting them into rats and mice. After periods vary-

[1] *Ges. Dtschr. Naturf. u. Arzte*, 1910.

[2] *Scientific Memoirs of the Government of India*, 1911, 42, p. 1.

[3] *Tr. Soc. Trop. Med. and Hyg.*, 1912, 5, p. 158.

[4] *Philippine Jour. Sc.*, 1909, 4, p. 403.

[5] *Loc. cit.* [6] *Jour. Exper. Med.*, 1912, 15, p. 292. [7] U.S. Government Reports, 1910.

[8] Paper read before the American Association of Pathologists and Bacteriologists, Philadelphia, 1912.

[9] *Australian M. Gazette*, 1912, 31, p. 209. [10] Personal communication. [11] *Loc. cit.*

ing from three weeks to several months, they were found to have turned into acid-fast rods."

Bayon attempts to support his position by reference to the experiments of Sanfelice with *Streptothrix alba* during which it was found that this organism can break up into cultivable acid-fast rods in the animal body.

Before Bayon's work Rost,[1] Williams,[2] Kedrowski,[3] and others had cultivated organisms other than acid-fast rods from leprous lesions and in the course of our investigations such organisms have come to light. Reference to these will be made later. The work of Rost and Williams is considered by them to show that the organism of leprosy (called *Streptothrix leproides*) is "an extremely pleomorphic streptothrix which under certain circumstances may be: (1) a non-acid-fast streptothrix with interlacing filaments; (2) a non-acid-fast diphtheroid bacillus, which is in reality a streptothrix, and capable of becoming acid-fast under certain defined conditions; (3) a definite acid-fast filamentous streptothrix, and (4) an acid-fast bacillus which is the broken-down stage of a streptothrix."

Now what is the explanation of these astounding findings? In the light of our own work it appears to be simple. We have met with the diphtheroid of Kedrowski in leprous lesions and have had no difficulty in obtaining it in pure culture, which we have by us at this writing, many generations from the parent culture. It has no tendency to become acid-fast either in the most diverse culture media or in the animal body. We regard it as a distinct organism and can find no evidence that it is etiologically related to leprosy. It is closely related to the well known group of diphtheroids (*B. pseudo-diphtheriticus*, *B. xerosis*, *B. gangosae*, etc.) which can be cultivated from various sources.

The branching filamentous and interlacing non-acid-fast and acid-fast streptothrices we have never found during a careful and exhaustive bacteriological examination of 29 cases of leprosy, and we are prone to consider it a contaminator.

To any who object to such explanations we would ask this question: Why is it that cultures obtained from leprous lesions, and containing one or both of these organisms, if injected into

[1] *Loc. cit.* [2] *Indian Med. Gazette*, 1911, 46, p. 249. [3] *Loc. cit.*

animals "turn into acid-fast rods"? We also ask: Why is it again, when the organisms are "recovered" from the animal, that no medium in the wide repertoire of the bacteriologist can persuade them to assume again their supposed normal streptothrical character when outside of the tissues? The answer is again simple. By passing the culture through the animal body one gets rid of the diphtheroid and streptothrical contaminators and recovers in pure culture what before was mixed growth, namely, the acid-fast bacillus of which we think there are two distinct species, the strictly parasitic and much more delicate of these being exceedingly apt to elude all but the most careful and special technic for its recognition where it is associated with another acid-fast species.

Such an explanation is in entire accord with the experience of all bacteriologists who, as is well known, commonly employ the device of passing cultures through animals in order to separate and secure in pure growth some particular species.

The bewildering number of "stages" in the supposedly single organism of the writers above mentioned presupposes such a sweeping change in all our ideas of biological analogy that only a most cautious and critical attitude is permissible when discussing these theories.

AUTHORS' RECENT RESEARCHES.

In the course of the work we have attempted the cultivation of the Hansen bacillus from 29 cases of leprosy and have succeeded in isolating an acid-fast bacillus from 22 of these cases. The chromogenic variety (Clegg) was recovered from 14 of these cases while eight yielded a non-chromogenic acid-fast bacillus which thus far has refused to produce pigment or multiply *in vitro* on ordinary laboratory media.

For many generations the subplants both of the chromogenic and of the non-pigment-producing forms have each remained well within the morphological variations of a species and have in general maintained pretty closely the morphology of the Hansen bacillus as we know it in the human lesion.

In the 14 cases above mentioned, the acid-fast culture recovered has eventually undergone a marked change in morphology and

cultural features[1] after which it could be propagated upon the ordinary laboratory media. These chromogenic cultures correspond to those first isolated by Clegg.

In the eight cases referred to, the non-chromogenic culture, although behaving much as did the Clegg chromogenic bacillus for the first two or three months under artificial growth conditions, has refused to alter in a similar manner. This bacillus grows well on an amino-acid medium but, unlike the Clegg culture, will not multiply on ordinary laboratory foodstuff.

Since the chromogenic culture behaved much in the same manner during the first three or four months under artificial cultivation, we have looked for a similar change to occur eventually for this "slow-growing" strain. It would seem, however, that it will not do so, as the period of parasitism experienced by the cultures which subsequently became chromogenic and vegetative has long passed. The oldest culture of this slow-growing, non-chromogenic bacillus has now been under cultivation for more than two years, but has attracted special attention only in the past nine months, because it persists in refusing to alter in any respect, though more than 50 generations removed from the parent stem. We have carried out exact studies (*vide infra*) to ascertain the minimum amount of amino-acid papulum necessary to keep this strain growing and have failed to do so upon any medium known to us except that containing split products of protein digestion.

In view of the delicate character of the growth of this organism and its conformity more to our ideas of a parasitic species than the chromogen, and thinking that the chromogenic strain in certain cases might possibly be mixed with it and thus account for the production of characteristic lesions in the monkey by Duval and Couret working with the Clegg type of culture, we have replated these cultures and find that the particular culture employed actually did contain the slow-growing non-chromogenic bacillus in symbiosis with, and overgrown by, the more vigorous chromogenic form.

In view of the significance attached by us at present to this non-pigment-producing strain which was first isolated from leprous

[1] *Arch. Inter. Med.*, 1911, 7, p. 230.

lesions and described by one of us (Duval) in 1910, we have thought best briefly to describe it again formally and to place cultures in the hands of other competent workers so that its exact status and importance may be settled.

Description of Organism.

The following brief résumé of the bacillus now being specially studied by us is here presented for comparison and reference. (For detailed description see Duval, *Jour. Exper. Med.*, 1910, 7, p. 649.)

Source of culture: subcutaneous leprosy nodule.

Date of isolation: November 10, 1909.

Morphology.—Vegetative cells on amino-acid medium at 37° C. These vary from short plump to long slender rods. The chromatin is irregularly placed, often resulting in a beaded and bipolar effect. Clear non-staining areas are in consequence also irregularly disposed.

Limits of size: $2–5\,\mu \times 0.2–0.4\,\mu$.

Endospores absent.

Flagella absent.

Capsule absent.

Involution forms: These may be seen in symbiotic cultures, in old pure cultures, and in cultures where the placental extract is diluted with other media (*vide infra* under "Special Methods of Cultivation").

Staining reactions: Takes the ordinary basic stains, is gram positive and acid-fast.

Cultural features.—(1) On amino-acid agar stroke culture is of scanty growth, moist, filiform, flat, glistening, smooth, and somewhat opaque.

Chromogenesis: None.

(2) In placental extract: surface growth, none under ordinary circumstances (floats are at the present writing being tried).

Clouding slight.

Sediment: moderately abundant.

Pathogenicity.—(See section on animal experiments.)

Brief characterization.—A gram positive and acid-fast, non-motile, moderately slender bacillus growing only on special media and generally presenting in culture the morphology of *B. diphtheriae.*

As to the inability of this bacillus to grow on ordinary media it may be said that we have undertaken exact experiments to determine the minimum amount of amino-acid foodstuff (placental extract) necessary to grow it upon solid media. Dilutions were made as follows:

Placental	extract	1	part;	3 per cent sterile agar		1	part.
"	"	1	"	nutrient	agar	2	parts.
"	"	1	"	"	"	3	"
"	"	1	"	"	"	4	"
"	"	1	"	"	"	5	"
"	"	1	"	"	"	6	"
"	"	1	"	"	"	7	"
"	"	1	"	"	"	8	"
"	"	1	"	"	"	9	"
"	"	1	"	"	"	10	"

Transplants from the original medium (placental extract 1 part, sterile agar 1 part) to the other dilutions showed that the organism will grow upon 1 part in 7 of placental extract with nutrient agar but will not multiply upon 1 part in 9 or 10.

SPECIAL METHODS OF CULTIVATION.

The initial multiplication of both the acid-fast strains referred to in this paper is accomplished with comparative ease provided that the bits of leprous tissues transferred are treated in such a way that the protein moiety is split into its dissociate products.

This action upon the protein of the removed leprous lesions may be accomplished in the following ways: (1) by seeding the tissue transplants with some one of the putrefactive bacteria or with any species capable of hydrolizing the tissues; (2) by saturating the removed tissue bits with 1 per cent trypsinized albumen solution; (3) by transferring the leprous material directly to a medium containing the products of protein digestion (placenta-extract agar).

With any of these methods the acid-fast bacilli in the removed lesions will multiply and continue to do so as long as these products are present, which of course is permanently in the case of the medium last mentioned.

In several instances we have failed to obtain any growth of acid-fast bacilli from leper patients. In one such instance the diphtheroid non-acid-fast organism (Kedrowski) was cultivated from the lesions. In these cases it may be mentioned that no acid-fast bacilli were demonstrable in stained smear preparations from the removed and macerated bits of tissue, although clinically the patients were regarded as typical lepers.

While perhaps the hydrolizing method offers the most certain means of obtaining the initial multiplication of the acid-fast bacilli, the placenta-extract agar is to be preferred since, especially in the case of the non-chromogenic bacillus, it does not necessitate replating and minimizes the chance of contamination. In fact we have found that even where the tissue is first hydrolized the addition of an amino-acid solution, such as placental juice, is distinctly advantageous, especially if glycerin is added, as the latter holds in check the growth of the hydrolizer.

When a hydrolizer alone is used continued multiplication is attended with the greatest difficulty as soon as the original split

products are exhausted, and even though placental extract or other amino-acids are substituted at this stage the growth activity is slower than in the initial period, taking from three to four weeks to attain its maximum.

Experimental Lesions with Different Leprosy Cultures.

In the course of the present study we have employed in our experiments 42 rabbits, 48 guinea-pigs, 35 white mice, 14 white rats, and two monkeys.

To determine if possible whether gross or microscopic differences exist for the experimental lesion induced by the two varieties of acid-fast bacilli which have been isolated and cultivated from the human leprous lesion, or whether the experimental lesions induced with either strain are similar to the human lesion or to those produced by the well known saprophytic acid-fast species, rabbits, guinea-pigs, and rats were injected with graduated doses of the following cultures, namely, five chromogenic leprosy cultures (Clegg, Duval, Brinkerhoff, Currie, and Bayon respectively), the non-chromogenic slow-growing lepra culture, and two representative saprophytic species (Mueller's grass bacillus and the bacillus of timothy hay).

The rabbits were inoculated intravenously, using 5 c.c. of a heavy homogeneous emulsion of the various cultures named. The animals received in all four injections at weekly intervals and were killed three months after the first inoculation. Similar experiments were carried out upon a series of guinea-pigs and white rats. In the series of guinea-pigs two injections were administered subcutaneously at intervals of 14 days, using 2 c.c. of a heavy homogeneous suspension for the first, and 4 c.c. for the second injection. The rats received intraperitoneally the same quantity of culture and number of injections.

The rabbits showed at autopsy microscopic lesions in the lungs, liver, spleen, and kidneys, irrespective of the culture employed, though macroscopic lesions were not demonstrable in all of the organs mentioned. The most marked gross lesions occurred in the rabbit injected with the bacillus of timothy hay and the chromogenic lepra cultures (Currie and Bayon). The most pronounced

and extensive lesions occurred in the rabbit which had received injections of the bacillus of timothy hay. In general the lung lesions resemble on naked eye appearance the miliary tubercle, while lesions in the liver are larger and of a somewhat different character. Here they resemble small healed gummata, with centers composed of a dry granular salmon-colored material surrounded by a dense tough zone of fibrous tissue.

The rabbits which received injections of the non-chromogenic culture of *B. leprae* showed no macroscopic evidence of lesions; however, the sections from the liver and spleen reveal definite areas in which the nuclei of the parenchymatous cell are broken and fragmented and the whole area crowded with acid-fast bacilli. For the most part the bacilli are scattered, with here and there dense collections contained within large cells and resembling the so-called leprosy globi. Although similar in some respects to the human leprous lesion in the internal organs and to those induced in the Japanese dancing mouse (Duval), they lack the histological picture (proliferative type of lesion) which characterizes the human leprous nodule.

The guinea-pigs were killed four months after the date of the first inoculation and in no case were there detected gross lesions except at the site of inoculation. In the animals which received the non-chromogenic leprosy culture there existed at the site of the second inoculation a small fibrous nodule, approximately the size of a split pea, which on microscopic examination contained a moderate number of acid-fast bacilli for the most part extracellular. The cell picture was not entirely characteristic of the human subcutaneous leprous nodule; however, it did resemble it in that there was no caseation and it contained many cells of the epithelioid type.

The guinea-pig which received Clegg's chromogenic lepra culture showed at the site of the second inoculation a fluctuating nodule the size of a marble. This area when opened discharged a thick, creamy yellow necrotic material and was found to contain enormous numbers of acid-fast bacilli in pure culture. The animal showed no microscopic lesions in the internal organs.

The series of white rats revealed no macroscopic lesions at

autopsy. However, small microscopic foci of lymphoid and plasma cells associated with scattered and clustered acid-fast bacilli were demonstrable on microscopic examination in sections from the omentum, spleen, and liver in one rat which had received the non-chromogenic lepra culture.

In general it may be stated that macroscopically the lesions produced in rabbits by intravenous injection do not differ greatly for any of the species of acid-fast cultures employed, unless it be that the chromogenic culture produces lesions which appear earlier and are more localized. With this culture the lung usually shows the most extensive change in the form of grayish-white discrete foci indistinguishable from miliary tubercles except possibly for the absence of necrosis. Microscopically the cell picture or relation of the bacilli with respect to the cells is not sufficiently distinctive of any culture employed to warrant more than a tentative differentiation. Although relative histological differences are detected for the experimental lesion, the difference is largely one of degree.

Suffice it to say that from our studies so far upon the experimental animals there is no absolute differentiation of the lesion induced by any given strain or species of acid-fast organism, excluding, of course, the tubercle family. A comparative study of the experimental lesions produced by the various acid-fast species is at present in progress by Dr. Couret in collaboration with one of us (Duval). Lesions are as readily induced experimentally with some of the well known saprophytic species as they are induced with either the infested leprous tissue or with lepra culture.

The five rabbit protocols here given are typical of the entire series and represent the results obtained by intravenous injections of the various cultures employed. An examination of these records will show the character and distribution of the induced lesions. All the animals of this series received at the same time and under the same conditions four doses of the respective cultures mentioned in the protocols.

Experiment 20. Rabbit A: Black female rabbit which had received four intravenous injections (ear vein) of an emulsion of *B. leprae* (Honolulu) on February 28, March 31, April 11, and April 22, respectively. The dose at each injection was approximately four billion bacilli. On April 14, the rabbit was bled from the heart and 4 c.c.

of blood removed. The animal steadily lost weight and died three months after the date of the first inoculation.

Gross examination.—The mesentery contains small firm nodules with small yellowish centers, ranging from 1 to 2 mm. in diameter. Several of the retroperitoneal lymph nodes are enlarged and on section show numerous yellow-colored necrotic areas. Smears from the necrotic material show innumerable scattered and dense colony masses of acid-fast bacilli. The lungs show extensive areas of hemorrhage and small well defined areas of consolidation. The peribronchial lymph nodes are enlarged but on section show nothing remarkable. On the right and left ventricles of the heart just beneath the epicardium are several small discrete yellowish areas similar to those in the mesentery. The liver contains many partially fibrosed gummatoid areas, the largest measuring 6 mm. in diameter. These areas in the liver are sharply defined and consist of an outer pearly gray translucent zone 1 mm. in thickness, and a central necrotic area which is of an orange-yellow color. Smears prepared from these necrotic centers show innumerable acid-fast bacilli. The other organs appear normal.

Microscopic examination.—The smaller lesions are made up of lymphoid and plasma cells and contain enormous numbers of acid-fast bacilli. In the more advanced lesions the central portion is composed of broken and fragmented nuclei, showing, however, no inflammatory reaction, while the lesion at the periphery is composed of epithelioid cells and fibroblastic elements forming a fairly dense encapsulating zone.

Rabbit B: Gray female rabbit which had received intravenously into the ear veins four injections of an emulsion of *B. leprae* (non-chrom.). The dose in each instance was approximately four billion bacilli which was injected on the following dates: February 28, March 31, April 11, and April 22. On May 14 the rabbit was bled and 4 c.c. of blood removed. The animal at this time was well nourished, vigorous, and showed no evidence of disease. Two months and a half after the first injection the rabbit was killed and at autopsy showed no demonstrable gross lesions except in the liver, where an occasional grayish-white poorly defined lesion was detected. Scrapings from these foci showed numerous acid-fast bacilli.

Microscopic examination.—Sections from the liver, spleen, and kidney show small areas of broken and fragmented cells and polymorphonuclear leukocytes and enormous collections of acid-fast bacilli. In no instance is there any fibrous tissue proliferation or presence of the epithelioid type of cell.

Rabbit C: Gray male rabbit which had been given four intravenous injections of an emulsion of *B. leprae* (Bayon) on the same dates as for the other animals of the series. The dose was approximately four billion bacilli. On April 14 the rabbit was bled from the heart and 4 c.c. of blood removed. The rabbit was killed three and one-half months after the first inoculation and at the time was well nourished and showed no evidence of disease.

Gross examination.—No gross lesions are detected except in the liver, where there is an occasional grayish-white area somewhat necrotic in the center. Smear preparations from this material shows enormous numbers of acid-fast bacilli.

Microscopic examination.—The sections from the various organs are negative except for the liver. Here the lesion is composed of lymphoid and epithelioid cells in and among which are numbers of acid-fast bacilli.

Rabbit D: A yellow and white female rabbit which had received intravenously on February 28, March 31, April 11, and April 22 four billion bacilli of the culture

bacillus of timothy hay. On April 14 the rabbit was bled from the heart and 4 c.c. of blood removed. The animal was killed three and one-half months after the first inoculation.

Gross examination.—At autopsy the lungs, liver, spleen, kidney, mesentery lymph nodes, mesentery, and omentum contain innumerable raised grayish-white necrotic foci ranging from pin-point to 2 mm. in diameter. The resemblance of the lesions in the lungs to miliary tubercles is striking. Acid-fast bacilli exist in these lesions in small numbers.

Microscopic examination.—The young lesions are composed of necrotic tissue, moderate number of polynuclears and giant cells, while older nodules show extensive necrosis, epithelioid formation, and infiltration with acute inflammatory elements.

Rabbit E: Black male rabbit which had received on four occasions an intravenous injection of *B. leprae* (Clegg). The dose was approximately four billion bacilli. The animal was killed three and one-half months after the first injection.

Gross examination.—In the ears where the injections had been made there is a marked phlebitis and thickening of the surrounding tissues. Smears from this location show enormous numbers of acid-fast bacilli which are for the most part in dense clusters or globi. On the left ventricle of the heart is a small hard white patch about the size of the head of a pin which also contain numerous acid-fast bacilli. This patch is well circumscribed and shows no evidence of necrosis. The other organs appear normal.

Microscopic examination.—The lesions are distinctly proliferative in character and occur in the liver, spleen, and kidney. The histological picture is indistinguishable from the human leprous nodule. In general they consist of a dense mosaic of epithelioid cells, interspersed with lepra cells containing globi.

Rabbit F: A white and black rabbit was injected intravenously on four separate occasions (February 28, March 31, April 11, and April 22) with approximately four billion bacilli of *B. leprae* (Currie). On April 14 the rabbit was bled from the heart and 4 c.c. of blood removed. The animal was well nourished and showed no evidence of disease. At autopsy gross lesions in the form of discrete areas 1 to 2 mm. in diameter are detected in the liver, spleen, and kidney. The other organs are apparently negative.

Microscopic examination.—The lesions do not differ in character from those described for Rabbit E.

Serological Tests with Different Strains of Organisms.

With the view of determining a possible relationship between the acid-fast chromogen, the non-chromogenic acid-fast, and the saprophytic chromogenic species, Dr. William H. Harris and John A. Lanford of the Department of Pathology have carried out an exhaustive serological study, which will shortly be published. A series of rabbits were immunized with the respective cultures and a comparative study carried out upon the immune sera for the detection of specific antibodies. Realizing the difficulty of immunizing against the acid-fast group the animals were subjected

to a long period of treatment, administering large doses intravenously at weekly intervals over a period of three months.

In addition the blood from a series of 20 cases of leprosy was also tested for specific antibodies to determine if possible a specificity for any given culture isolated from the same case or from other cases of leprosy. In performing these serum tests the agglutination reaction and the complement-binding tests, using a culture antigen, were employed.

Their results show that the serological tests with the blood of lepers has not established an etiological rôle for either type of acid-fast organism recovered from the leprous lesion. The agglutination reaction with the lepers' blood rarely gives a positive reaction in dilution of 1:50 with the separated Hansen bacilli obtained from the human nodule, while in the majority of cases a reaction is not obtained above a dilution 1:10. On the other hand, many of the tubercle family and the acid-fast saprophytes react equally as well and not infrequently in higher dilutions (see Table 1). The Wassermann reaction with culture antigen utterly fails to show anything specific for the two varieties of culture in so far as the human serum is concerned. However, the serum reaction of animals immunized against the various acid-fast species has served to separate into three distinct groups, namely, the chromogenic culture of leprosy (Group I), the non-chromogenic culture of leprosy (Group II), and the chromogenic saprophytic acid-fast species (Group III).

The reaction with specific immune serum establishes the fact that there is a difference between the non-chromogenic and the chromogen leprosy cultures. Furthermore the serum reaction indicates no relation between these two strains and any saprophytic species.

The following tables are from the series of leprosy cases mentioned above and are typical of the entire series, including examples of reactions both in high and in low dilutions.

The tests consisted (1) of the reactions of the patients' own blood with 15 strains of acid-fast bacilli; (2) of the agglutination reactions and complement fixation tests with specific immune sera.

In order to avoid repetition and the use of unnecessary space only three tables will be given. They represent the results of tests

made with the sera obtained from cases of human leprosy and with specific immune sera of rabbits.

TABLE 1.

AGGLUTINATION REACTIONS WITH SERUM OF LEPERS.

LEPROSY CASE	CULTURE (SUSPENSION)	SERUM DILUTION	RESULT 1 Hour	RESULT Final	REMARKS
II. Brunner. Trophic type. Duration 10 years	1. Non-chrom	1:10	+	+	Positive reaction with chromogenic leprosy cultures generally in higher dilution than with other species. This case had received several injections of the protein extract of Culture 3 (chromogen, Duval).
	2. Chrom. (Clegg)	1:160	+	+	
	3. Chrom. (Duval)	1:160	+	+	
	4. Chrom. (Currie)	1:160	+	+	
	5. Chrom. (Brinkerhoff)	1:10	+	+	
	6. Chrom. ("Hawaii")	1:80	+	+	
	7. Chrom. (Bayon I)	1:80	+	+	
	8. Tubercle (human)	1:40	+	+	
	9. Tubercle (bovine)	1:10	+	+	
	10. Tubercle (avian)	1:10	+	+	
	11. Timothy hay (dry)	1:10	+	+	
	12. Mueller's No. 1	1:10	+	+	
	13. Mueller's No. 2	1:10	+	+	
	14. Korn	1:10	+	+	
	15. Karlinski	1:10	+	+	
VIII. Fritz. Nodular type. Duration 5 years	1. Non-chrom	1:80	+	+	All acid-fast cultures used agglutinate with this serum except the "dry" growers (B. timothy hay and Karlinski).
	2. Chrom. (Clegg)	1:80	+	+	
	3. Chrom. (Duval)	1:80	+	+	
	4. Chrom. (Currie)	1:10	+	+	
	5. Chrom. (Brinkerhoff)	1:40	+	+	
	6. Chrom. ("Hawaii")	1:40	+	+	
	7. Chrom. (Bayon)	1:20	+	+	
	8. Tubercle (human)	1:20	+	+	
	9. Tubercle (bovine)	1:80	+	+	
	10. Tubercle (avian)	1:40	+	+	
	11. Timothy hay (dry)	1:10	−	−	
	12. Mueller's No. 1	1:70	+	+	
	13. Mueller's No. 2	1:40	+	+	
	14. Korn	1:20	+	+	
	15. Karlinski	1:10	−	−	
XIV. Moore. Maculo-anesthetic type. Duration 4 years	1. Non-chrom	1:40	+	+	Good agglutinations throughout the series with this serum.
	2. Chrom. (Clegg)	1:40	+	+	
	3. Chrom. (Duval)	1:80	+	+	
	4. Chrom. (Currie)	1:80	+	+	
	5. Chrom. (Brinkerhoff)	1:80	+	+	
	6. Chrom. ("Hawaii")	1:40	+	+	
	7. Chrom. (Bayon)	1:40	+	+	
	8. Tubercle (human)	1:40	+	+	
	9. Tubercle (bovine)	1:40	+	+	
	10. Tubercle (avian)	1:40	+	+	
	11. Timothy hay (dry)	1:40	+	+	
	12. Mueller's No. 1	1:80	+	+	
	13. Mueller's No. 2	1:20	+	+	
	14. Korn	1:70	+	+	
	15. Karlinski	1:50	+	+	
XII. Chevalier. Mixed type. Duration 8 years	1. Non-chrom	1:10	−	+	Low agglutinations throughout except with 8 and 13. This case received subcutaneously the protein extract of 3 (chrom. Duval) over a period of one year.
	2. Chrom. (Clegg)	1:40	+	+	
	3. Chrom. (Duval)	1:40	+	+	
	4. Chrom. (Currie)	1:10	+	+	
	5. Chrom. (Brinkerhoff)	1:40	+	+	
	6. Chrom. ("Hawaii")	1:10	+	+	
	7. Chrom. (Bayon)	1:10	+	+	
	8. Tubercle (human)	1:150	+	+	
	9. Tubercle (bovine)	1:40	+	+	
	10. Tubercle (avian)	1:10	+	+	
	11. Timothy hay (dry)	1:10	+	+	
	12. Mueller's No. 1	1:80	+	+	
	13. Mueller's No. 2	1:20	+	+	
	14. Korn	1:70	+	+	
	15. Karlinski	1:50	+	+	

TABLE 1.—*Continued.*

Leprosy Case	Culture (Suspension)	Serum Dilution	Result 1 Hour	Result Final	Remarks
VI. Amelia. Tubercular type. Duration 3 years	1. Non-chrom	1:80	+	+	Avian tubercle and Mueller's grass bacillus react equally as well with this serum as the non-chrom. leprosy culture.
	2. Chrom. (Clegg)	1:60	+	+	
	3. Chrom. (Duval)	1:40	+	+	
	4. Chrom. (Currie)	1:40	+	+	
	5. Chrom. (Brinkerhoff)	1:40	+	+	
	6. Chrom. ("Hawaii")	1:10	+	+	
	7. Chrom. (Bayon)	1:40	+	+	
	8. Tubercle (human)	1:40	+	+	
	9. Tubercle (bovine)	1:10	—	—	
	10. Tubercle (avian)	1:80	+	+	
	11. Timothy hay (dry)	1:10	—	—	
	12. Mueller's No. 1	1:80	+	+	
	13. Mueller's No. 2	1:10	+	+	
	14. Korn	1:40	+	+	
	15. Karlinski	1:10	—	—	
VII. Pablo. Maculo-anesthetic type. Duration 3 years	1. Non-chrom	1:10	+	+	All cultures react in low dilutions except with Mueller's grass bacillus No. 1 which gives the highest reaction.
	2. Chrom. (Clegg)	1:10	+	+	
	3. Chrom. (Duval)	1:20	+	—	
	4. Chrom. (Currie)	1:10	+	+	
	5. Chrom. (Brinkerhoff)	1:40	+	+	
	6. Chrom. ("Hawaii")	1:10	—	+	
	7. Chrom. (Bayon)	1:10	+	+	
	8. Tubercle (human)	1:10	+	+	
	9. Tubercle (bovine)	1:10	+	+	
	10 Tubercle (avian)	1:10	+	+	
	11. Timothy hay (dry)	1:10	—	+	
	12. Mueller's No. 1	1:60	+	+	
	13. Mueller's No. 2	1:10	+	+	
	14. Korn	1:10	+	+	
	15. Karlinski	1:10	+	+	

TABLE 2.

AGGLUTINATION REACTION WITH ANTI-LEPROSY SERA.

Immune Serum	Culture Suspension	Dilution of Sera	Results 1 Hour	Results Final	Remarks
3. Chrom. (Duval)	1. Non-chrom	1:10	—	—	
	2. Chrom. (Clegg)	1:10	+	+	
	3. Chrom. (Duval)	1:160	+	+	
	4. Chrom. (Currie)	1:10	+	+	
	7. Chrom. (Bayon)	1:10	—	+	
	11. Timothy hay (dry).	1:10	—	—	
1. Non-Chrom	1. Non-chrom	1:150	+	+	
	2. Chrom. (Clegg)	1:10	+	+	
	3. Chrom. (Duval)	1:80	+	+	
	4. Chrom. (Currie)	1:10	+	+	
	7. Chrom. (Bayon)	1:10	+	+	
	11. Timothy hay (dry)	1:10	+	+	
2. Chrom. (Clegg)	1. Non-chrom	1:10	—	—	
	2. Chrom. (Clegg)	1:50	+	+	
	3. Chrom. (Duval)	1:10	+	+	
	4. Chrom. (Currie)	1:10	+	+	
	7. Chrom. (Bayon)	1:10	+	+	
	11. Timothy hay (dry)	1:10	—	—	
4. Chrom. (Currie)	1. Non-chrom	1:10	—	+	
	2. Chrom. (Clegg)	1:40	+	+	
	3. Chrom. (Duval)	1:20	+	+	
	4. Chrom. (Currie)	1:320	+	+	
	7. Chrom. (Bayon)	1:10	+	+	
	11. Timothy hay (dry)	1:10	—	+	

TABLE 2.—*Continued.*

Immune Serum	Culture Suspension	Dilution of Sera	Results: 1 Hour	Results: Final	Remarks
5. Chrom. (Bayon)	1. Non-chrom.	1:10	+	+	
	2. Chrom. (Clegg)	1:20	+	+	
	3. Chrom. (Duval)	1:10	+	−	
	4. Chrom. (Currie)	1:10	+	−	
	7. Chrom. (Bayon)	1:190	+	+	
	11. Timothy hay (dry)	1:10	+	+	
6. Timothy hay (dry)	1. Non-chrom.	1:10	+	+	
	2. Chrom. (Clegg)	1:10	+	+	
	3. Chrom. (Duval)	1:10	+	+	
	4. Chrom. (Currie)	1:10	−	+	
	7. Chrom. (Bayon)	1:80	+	+	
	11. Timothy hay (dry)	1:320	+	+	

TABLE 3.

Complement Fixation Tests.

Rabbit Immune Serum	Culture Antigen	Results: 1 Hour	Results: 24 Hours	Remarks
1. Non-chromogen	1. Non-chrom.	++	++	When larger amount of antigen was employed all are −+
	2. Chrom. (Clegg)	−−	−−	
	3. Chrom. (Duval)	−−	−−	
	4. Chrom. (Currie)	−−	−−	
	7. Chrom. (Bayon)	−−	−−	
	11. Timothy hay (dry)	−−	−−	
2. Chromogen (Clegg)	1. Non-chrom.	−−	−−	
	2. Chrom. (Clegg)	−+	−+	
	3. Chrom. (Duval)	−+	−+	
	4. Chrom. (Currie)	−+	−+	
	7. Chrom. (Bayon)	−+	−+	
	11. Timothy hay (dry)	−−	−−	
3. Chromogen (Duval)	1. Non-chrom.	++	++	When larger amount of antigen was employed all are ++
	2. Chrom. (Clegg)	++	+−+	
	3. Chrom. (Duval)	++	+−+	
	4. Chrom. (Currie)	++	+−+	
	7. Chrom. (Bayon)	++	++	
	11. Timothy hay (dry)	−−	−−	
4. Chromogen (Currie)	1. Non-chrom.	−−	−−	
	2. Chrom. (Clegg)	−−+	−−+	
	3. Chrom. (Duval)	−−+	−−+	
	4. Chrom. (Currie)	−−+	−−+	
	7. Chrom. (Bayon)	−−+	−−+	
	11. Timothy hay (dry)	−−	−−	
7. Chromogen (Bayon)	1. Non-chrom.	−++	−+	
	2. Chrom. (Clegg)	−−+	−−+	
	3. Chrom. (Duval)	−−+	−−+	
	4. Chrom. (Currie)	−−+	−−+	
	7. Chrom. (Bayon)	++	++	
	11. Timothy hay (dry)	−−	−−	
11. Timothy hay (dry)	1. Non-chrom.	−−	−−	When larger amount of antigen was employed all are −−+
	2. Chrom. (Clegg)	−−	−−	
	3. Chrom. (Duval)	−−	−−	
	4. Chrom. (Currie)	−−	−−	
	7. Chrom. (Bayon)	−−	−−	
	11. Timothy hay (dry)	++	++	

These rabbits received five injections of viable cultures, approximately four billion, at each inoculation and at weekly intervals over a period of four months.

++ equals very positive; −+ equals weakly positive; −− equals negative.

GENERAL DISCUSSION.

The following is the origin and number of the cultures studied by us during the course of the present investigation: (1) Clegg (four cultures), (2) Brinkerhoff (one culture), (3) Currie (two cultures), (4) Bayon (two cultures), (5) "Hawaii" (five cultures), (6) Duval (fourteen cultures), (7) Rost and Williams (one culture).

From our work with these the following considerations occur to us.

There can be little doubt that Clegg with amebae and their symbionts obtained multiplication *in vitro* of both strains of the acid-fast bacilli herein referred to, for, as already pointed out, the non-chromogenic strain will multiply in the test-tube provided the tissue bits are hydrolized. Clegg undoubtedly accomplished this through the symbionts which were associated with the amebae. His culture sent to us, which he obtained pure by heating, grew readily upon ordinary laboratory media, and contained only the chromogenic bacillus. We believe the non-chromogenic parasitic variety, if originally present, ceased to multiply after the mixed culture was transferred to ordinary media.

The occurrence of the chromogenic bacillus of Clegg in leprous lesions, especially where it occurs in the internal organs, is difficult to account for if we are to accept that it is a simple saprophyte. On the other hand, how are we to explain the occurrence of what is apparently another distinct organism in the lesions of leprosy? Are we dealing with two etiological factors, or is one the causal agent and the other an associated commensal, or do they both represent stages of the same species?

With this question in mind we have gone back over some of the cases previously examined by us (1911) to determine if possible what percentage yields the chromogenic culture and what proportion yields the non-chromogenic type or whether in any case the two are encountered. For this purpose we selected five cases of leprosy from which the chromogenic bacillus had previously been isolated. The nodules were removed with every care to avoid against extraneous contamination. Each nodule was divided into two portions, one part being hydrolized with *B. subtilis* while the other was treated with placental juice. These cultures were incubated

at 37° C. over a period of two months and examined at frequent intervals to note the character and behavior of the growth. In no case and at no time were we able to detect other than acid-fast bacilli (except of course in the tubes where *subtilis* had been added), and these acid-fast bacilli multiplied steadily and retained their acid-fastness throughout the entire period of growth. The acid-fast cultures obtained by means of the hydrolizer were subsequently obtained pure by plating on placental-extract agar. Cultures of acid-fast bacilli were recovered from all five cases. Two of these have taken on chromogenic properties and become culturally like the original isolation. The others show no tendency to alter in this respect, but conform to the description of the bacillus first described by Duval. From three of the earlier cases (1910) we have also recovered recently by plating on placental-extract agar an acid-fast culture which for months has grown slowly, is non-chromogenic, and refuses to multiply except on special media. In four other cases from which one of us (Duval) in 1910 isolated the chromogenic culture we have recently attempted a second isolation, but the cultures have all turned out to be chromogens.

It is, we repeat, hard to explain the occurrence in leprous lesions of this chromogenic acid-fast variety which in our experience with cases here is encountered more frequently than the non-chromogenic variety. This may possibly be explained by our present imperfect methods of cultivation. Curiously enough the chromogenic type, if we are to regard it as an extraneous organism, is always the same variety, i.e., a moist, rapidly growing bacillus when once it becomes accustomed to an artificial environment. We have compared the original cultures of Clegg with those isolated independently by workers in other parts of the world and find them, except for inconstant minor differences, identical. That this chromogenic species exists in the lesions of certain types of leprosy there can be no doubt, and that, too, in the lesions where the overlying skin is apparently intact as well as from the internal organs at autopsy, e.g., from the spleen.

It is interesting to note that the Clegg strain undergoes at times the most marked change in tinctorial and morphological features. It is relatively easy to obtain from this species a non-acid-fast

diphtheroid, an acid-fast beaded bacillus, and a diplococcoid acid-fast type. These cultures when grown upon an alkalin medium in the presence of symbionts are distinctly acid-fast, while the individual rods are indistinguishable from tubercle bacilli. On an acid medium in symbiosis with other bacteria they occur as non-acid-fast pleomorphic forms, many of which are distinctly diphtheroid in appearance. In pure culture on such media the individual bacilli are small acid-fast ovoid or coccoid rods occurring singly or in pairs. This wide variation in morphology and difference in staining properties might possibly account for the non-acid-fast diphtheroid "stages" described by European authors.

In the entire series of cases from which we have attempted the cultivation of the Hansen bacillus we have noted in but one case a non-acid-fast diphtheroid. This culture in our hands has failed to change into an acid-fast by passing it through rats or handling in any other manner. In other words, the non-acid-fast diphtheroid stage for the Hansen bacillus which has been described by Babes, Bordoni-Uffreduzzi, Kedrowski, and others has not occurred in our experience except in so far as the transitory changes in the Clegg culture above mentioned may be thus interpreted.

So far as the non-chromogenic, slow-growing bacillus is concerned, we believe careful attention is merited by it. In cultures it is always acid-fast and usually occurs as long and short beaded rods, although in a lesser degree the morphological change described above for the Clegg culture may be applied to this culture also. If it was not for the fact that this bacillus is always acid-fast it would be impossible to distinguish it morphologically from *B. diphtheriae*.

The presence of the chromogenic Clegg bacillus in the tissues of human lepers, together with the fact that disseminated lesions are induced experimentally which are histologically like those in human leprosy, led one of us (Duval) in a former publication to conclude that the chromogenic strain played an etiological rôle in the disease. In going back over some of the preparations from the animals employed we find them to contain the long beaded acid-fast rods in dense masses and also many more scattered bacilli which are shorter and less beaded (Clegg bacillus). These findings

were described in the paper referred to as indicating a transformation of the long slender beaded rods to diplococcoid forms, but by replating on placental media the culture employed we find it to be mixed, containing both strains, so that it is impossible to say without further work just what part the two types played in the production of lesions in the monkey. This view has been greatly strengthened by our recent results obtained by replating upon placental agar cultures from the original tryptophane fish agar formerly described by one of us (Duval). We find that in these the two varieties exist and it is therefore not improbable that the subplants of the earlier isolations upon amino-acid media would also yield both cultures.

We have not received the information we hoped for from animal experimentation. This is not surprising in view of the difficulty of inoculation experiments upon laboratory animals. The most that can be broadly stated is that, excepting *B. tuberculosis*, the lesions induced by acid-fast bacilli are not sharply differentiated. The general character of the lesions following injection of animals with the various acid-fast organisms may be briefly characterized as follows: (1) Leprosy cultures produce lesions proliferative in type without necrosis, and consist of an epithelioid cell matrix which is strongly suggestive of the human leprosy lesion. The contained bacilli are for the most part scattered and extracellular, though dense masses of organisms (globi) within large multinucleated cells occur; (2) saprophytic cultures (timothy hay, etc.) produce lesions which differ from the foregoing principally by the presence of sub-acute inflammation and extensive necrosis; (3) the experimental lesions of tuberculosis are too well known to call for remark. Even with avian cultures the lesion is distinctive enough not to confuse it with leprosy. The refractory character of laboratory animals to leprosy cultures must be borne in mind when attempting to interpret the results from experiments.

From the serological tests we have only succeeded in showing that the sera of highly immunized animals will as a rule react better to the immunizing strain than to other allied or to foreign acid-fast strains, but the reactions employing as an index either the agglutinins or the deviation of complement cannot be said to be

specific. The results in this regard are directly contradictory to those of Bayon,[1] who believed that by freezing and thawing the bacilli he could produce a satisfactory antigen which would enable him to differentiate his leprosy culture from other groups by means of the complement deviation test.

In this connection, however, attention should be drawn to the more specific character of the reaction obtained in lepers by Clegg,[2] Duval and Gurd,[3] and others who use the chromogenic leprosy cultures. A cutaneous reaction similar to that secured with tuberculin in tuberculous patients was obtained by these authors by means of injections with killed bacilli (chromogen Clegg) or the "leprosin" (protein extract) of the same. A marked constitutional reaction consisting of a rise in temperature (104° F.) and a distinct leukocytosis (15,000–24,000) was induced by the injections, together with lepra abscess formation at the site of inoculation. Acid-fast saprophytes (timothy hay) failed to give the reaction. It is possible that here we have a better means of separating the strain of leprosy cultures and that further work along these lines will yield more definite results.

SUMMARY.

From the leprous lesion two varieties of acid-fast bacilli may be cultivated, one a chromogenic pleomorphic organism which grows readily upon the ordinary laboratory media after it has become accustomed to a saprophytic existence; the other, a moist-growing non-chromogenic bacillus resembling tinctorially the tubercle bacillus, and morphologically the diphtheria bacillus, and multiplying only upon special media. The chromogenic strain, although hard to cultivate at first, subsequently grows profusely and rapidly upon a great variety of foodstuff, while the non-chromogenic strain is always difficult to cultivate and multiplies very slowly even in generations far removed from the parent stem.

The chromogenic culture may show a wide variation in morphology and its ability to retain the stain when subjected to decolorizing agents. At times and under certain conditions the individual rods are diphtheroid and non-acid-fast. The non-chromogenic culture

[1] *Loc. cit.* [2] *Phil. Med. Bull.*, 1910. [3] *Loc. cit.*

is always acid-fast and can be sharply differentiated from the chromogenic culture by its growth features.

CONCLUSIONS.

1. From a bacteriological study of 29 cases of leprosy we have isolated an acid-fast bacillus from 22 cases.

2. A chromogenic strain similar in all essentials to that described by Clegg was recovered from 14 cases.

3. Eight cases yielded an organism which is markedly different in its character from Clegg's bacillus and which will grow only on specially prepared media and refuses to become chromogenic.

4. In one case we have isolated a non-acid-fast diphtheroid bacillus corresponding to the organism described by Kedrowski.

5. We are unable to confirm the work of Rost, Williams, Bayon, and others who consider that *B. leprae* is a bacterium of such pleomorphism that it can be recognized as a diphtheroid, a streptothrix, and an acid-fast bacillus.

6. Animal experiments undertaken for the purpose of differentiating the acid-fast organisms and to fix their etiological status are not regarded by us as conclusive.

7. Serological tests, especially those performed with highly immune sera, have proved of some value and tend to show that Clegg's bacillus of leprosy is not related to the ordinary acid-fast chromogenic saprophytes, and that the non-chromogenic lepra culture of Duval is different both from Clegg's organism and from all other acid-fast bacilli.

8. The rôle played by the chromogenic bacillus of Clegg in the production of leprosy is as yet an unsettled question, although we are at present inclined to ascribe to it a minor if not a negligible part.

9. The non-chromogenic strain, while behaving according to most of our notions regarding a pathogenic organism, has likewise not up to the present been conclusively proved the cause of leprosy, although we are impressed with the probability of such a rôle being eventually attributed to it and consider that it deserves more serious attention than any organism so far cultivated from the human leprous lesion.

The
Journal of Infectious Diseases

PUBLISHED BY THE MEMORIAL INSTITUTE FOR INFECTIOUS DISEASES

VOL. II | *September 1912* | No. 2

IS TYPHOID FEVER A "RURAL" DISEASE?*

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1. INTRODUCTION.

There has long been a widespread belief that typhoid fever is a "rural" disease, i.e., a disease having its natural habitat in the country. On the other hand, bacteriology and epidemiology have proved that typhoid fever is a disease of contact, of crowding, and

* Received for publication May 2, 1912.

of infected food and drink—conditions far more common in city than in country. Obviously, so sharp a contradiction between a condition reputed to exist, and a condition required by theory, calls for careful investigation.

Paragraphs in the newspapers and magazines often advise persons about to visit the country to beware of typhoid infection, implying if not affirming that the dangers of such infection are greater in the country than in the city. And when the annual autumnal excess of typhoid fever, which occurs almost everywhere in northern latitudes, draws attention to this disease, it is frequently asserted, not only by newspaper and magazine writers, but also by medical men—and sometimes even by sanitarians—that this excess is due to the fact that many city dwellers have recently returned from vacations in the country, and have brought with them either typhoid fever or the germs of typhoid fever.

This explanation of some cases of autumnal typhoid is so obviously true that the forbidding term "vacation typhoid" has been coined and widely circulated and is now often accepted at its face value, even by persons who have given attention to the causation of typhoid fever, as satisfactorily accounting for most of the regular autumnal excess of that disease. This modern notion of "vacation" typhoid, moreover, is closely connected with an older theory that typhoid fever is pre-eminently a "rural" disease, a doctrine long maintained on good grounds by excellent authorities and powerfully supported by theories—such as Murchison's pythogenic theory -of the spontaneous origin of fevers like typhoid in manure-heaps, decaying organic matters, and filth of various kinds.

The rise of bacteriology and the decade of the great pathological discoveries have, however, disproved Murchison's and similar theories, and the simple faith of a former generation in filth and "fomites" as birthplaces for disease germs has been proved to be groundless. Meantime, the importance of contact, of carriers, of crowding, of promiscuity, and of public supplies of all sorts as sources and vehicles of infection, and the value of isolation of persons and supplies and of the absence of contact, of crowding, and of promiscuity as safeguards against infection have become generally admitted and recognized, until it is today almost impos-

sible to believe that the dangers of infection in the sparsely settled country are anywhere near as great as in the crowded city, or *a fortiori* that typhoid fever is today really a "rural" disease. This fact is strengthened and supported by considerations of bacteriology and epidemiology, for it is today impossible to believe that the germs of typhoid fever which must pass from person to person by contact or in food or drink, can be as widely or as effectively distributed by isolated carriers, isolated wells, or isolated milk supplies in rural districts, as by the city carrier necessarily coming into contact with far more people, or the public water or milk supply of the city, reaching as it does simultaneously hundreds or thousands, instead of units or tens or twenties.

And yet it is still often affirmed that "typhoid fever is a rural disease" and implied if not stated that city people who spend vacations in the country are more liable to contract typhoid fever there than they would be if they stayed in the city. We have here therefore, as already said, a flat contradiction between conditions reputed to exist and conditions according to modern ideas to be expected; and it is this contradiction which has seemed to us to require explanation, and has induced us to examine into the facts and to prepare the present paper.

The question at issue is simply *whether, in proportion to the population, typhoid fever is more prevalent over rural or over urban areas;* and for an answer we have turned to the mortality statistics of Massachusetts as a state having trustworthy records and one with which we are personally familiar. These statistics we have examined from various points of view with special reference to the question at issue. For control, we have made a similar though less minute study of the mortality statistics of a more rural contiguous state, New Hampshire, as well as those of Connecticut, another contiguous state which for various reasons is also instructive in this connection. Brief references have likewise been made to the mortality statistics of foreign countries, such as England and Wales, Scotland, and Germany.

Before presenting the methods and results of our statistical studies, we shall consider at some length the rise and the present state of opinion relating to typhoid fever as a rural disease; and in

another section, also in some detail, the common employment of the terms "rural" and "urban," as well as their true sanitary significance and proper scientific usage.

2. STUDIES AND STATEMENTS OF PREVIOUS WRITERS.

As far as we are aware, the earliest statistical study which led to the conclusion that typhoid fever is a rural disease was made by Dr. George Derby, secretary of the then newly organized State Board of Health of Massachusetts, and published in the Report of that Board for 1871. Dr. Derby's paper is entitled "The Causes of Typhoid Fever in Massachusetts" and is an elaborate statistical research covering the decennium, 1859–68. Dr. Derby's studies were based upon the mortality statistics of typhoid fever in all the "towns" of Massachusetts for that period. Because of the fundamental character of this paper, and the probability that from it has very largely come the present doctrine of typhoid fever as a rural disease, we shall quote from it somewhat freely:

"The first thing which strikes us on looking over this table is the apparently greater mortality from typhoid in the small towns. How great this difference is will appear from the following comparison:

TABLE SHOWING RELATIVE MORTALITY FOR TEN YEARS FROM TYPHOID FEVER IN PERSONS ABOVE FIVE YEARS OF AGE, IN THE LARGER AND SMALLER CITIES AND TOWNS.

	Population 1865 (All Ages)	Total Deaths from Typhoid in 10 Years	Average Number Persons Living Each Year to One Death	Average Number Deaths Each Year to 1,000 Persons Living
147 cities and towns of more than 2,000 inhabitants	1,044,294	7,888	1,323.90	0.755
184 towns of less than 2,000 inhabitants	213,468	2,539	840.75	1 189

"There can be no doubt that typhoid in Massachusetts is a disease of scattered communities rather than of crowded towns, of rural rather than of urban districts. In spite of the smaller mortality from all causes, typhoid is more destructive in the farming towns than in the manufacturing towns and the large cities" (pp. 118, 119).

Again, later in the same paper:

"Everyone familiar with the state knows that there is a very large number of towns with a population of from five to ten thousand, compactly built, with no water-supply except from wells, and no means of disposing of excrement except by privies, and we know from the registration returns that the people of these towns are more free from the pest of typhoid in proportion to population than the inhabitants of agricultural districts" (p. 166).

Again, from the letter of a Boston correspondent:

"A very considerable number of the cases of typhoid treated in Boston during the autumn originates in the country and at seaside places where families from the city have passed the summer" (p. 128).

This able and thorough study thus arrives at a definite conclusion that typhoid fever is chiefly a disease of communities of less than 2,000 inhabitants. Here is apparently one of the main sources of the "rural typhoid" doctrine.

The next study of the kind, so far as we have been able to discover, was a thorough and detailed statistical research, published by Dr. Samuel W. Abbott in the Report of the State Board of Health of Massachusetts for 1891, as a part of a paper on "The Geographical Distribution of Certain Causes of Death in Massachusetts." The first part of the section on typhoid fever, which covers the twenty-year period, 1871–90, deals with the relation of prevalence to density of population, by counties. The following summary table is an abridgment of that given on p. 822 of the report.

Counties Grouped According to Their Density of Population	Average Acres to Each Person	Mortality Rank from Typhoid Fever. The State = 100
I. County in which there is less than one acre to each inhabitant	0.07	91
II. Counties (5) having more than one but less than four acres to each inhabitant	2.10	101
III. Counties (8) having more than four acres to each inhabitant	6.40	107

Dr. Abbott concludes from this table that:

"The mortality from typhoid fever does not depend upon the density of population as the principal favoring condition for its spread. The most densely settled metropolitan district had a comparatively low mortality rate from this cause, while the group of sparsely settled counties had an average high ratio of mortality from typhoid fever. . . . ."

"Estimating the mortality of the densely settled districts as 1,000, the mortality of these three groups from typhoid fever was as follows:

The *dense* districts	1,000
The *medium* districts	1,109
The *sparse* districts	1,175

The analysis thus summarized has apparently furnished a basis for the doctrine that typhoid fever is chiefly a rural disease, because it is here shown, under the conditions considered, to be more prevalent in the *sparsely* settled counties. But while density studies

applied to small areas—as those of a New England "town"—may be reliable, the results from the comparatively large areas of counties are, in the light of the considerations presented in section 3 of this paper, open to serious criticism. In the county all grades of urban and rural life are included and merged together and *general* density figures cannot give any adequate indication of that local distribution of population which is the *sine qua non* of a study of urban vs. rural conditions.

Dr. Abbott, however, later in his study, does take up the separate towns considered with reference to density of population, and this mode of attack we believe to be much more effective than that just quoted. He bases his observations upon a table, similar to the one presented by Dr. Derby for 1859–68 in which the towns of Massachusetts are arranged according to their typhoid death-rates.

"An examination of the extremes reveals no very striking characteristics. But one town, Gosnold (the smallest in population), had no deaths from typhoid fever during the twenty years. This town comprises the Elizabeth Islands, lying at the mouth of Buzzard's Bay. Its population in 1880 was 152.

"The twenty towns having the lowest rank in the list are scattered throughout the state in ten of the counties, and, with the exception of one of the smaller cities (Waltham), are small towns. Twelve of this number had less than 1,000 inhabitants in each in 1880. There does not appear to be any point of special significance relative to these twenty towns.

"Of the twenty towns which hold the highest position upon the list, seventeen are in the four western counties and the western border of Worcester County. Dalton, with the highest ratio, is a manufacturing town (paper mills) among the Berkshire Hills, lying at an elevation of more than 1,000 feet above the sea-level. Ware, Brookfield, Great Barrington, and Westfield are also manufacturing towns of western Massachusetts. Ten of the remaining towns are small agricultural towns, having a population in each of less than 1,000, and their position may hence be regarded as having but little significance.

"The city of Holyoke, which had 498 deaths from typhoid fever in the twenty-year period, and ranked 203 in this list as compared with the state (100), is a manufacturing city of rapid growth. Its population in 1870 was 10,733; in 1880, 21,915, and in 1890, 35,637. The principal industry is the manufacture of paper" (p. 823).

A classification of towns by density of population is thus described:

"For the purpose of making a more intelligent classification I have therefore classified the towns into two general divisions of manufacturing or densely settled towns, and agricultural or sparsely settled towns.

"The former embraces 87 municipalities, including all the 28 cities. None of

this number had less than 2,000 inhabitants in each, and only 10 had less than 4,000; and each of these smaller towns had one or more villages having a comparatively dense population living in a district of limited size. The average population of each municipality in this group by the census of 1880 was 15,434. Most of them have a steadily increasing population, a large proportion of which consists of immigrants, either of European nativity or from the British North American provinces. In many of the towns of this class there is a rural population of considerable size living in those portions outside the more densely settled villages. The actual increase in population in the rural towns, for the period of twenty years, between the census of 1870 and that of 1890, a period nearly coincident with that under consideration, was $11\frac{1}{4}$ per cent, while that of the larger or manufacturing and urban districts was 70 per cent.

"The other group, of agricultural towns, 259 in number, has but few towns having 3,000 in each, and the average population of each in 1880 was 1,700. Their population is distributed over an average area of nearly 30 square miles for each township. The principal occupation of the people of these towns consists of dairy farming, market gardening, and other branches of agriculture. A few of these towns are summer resorts, either upon the sea-coast or in the inland region. Some of the towns of this general class have increased slightly in population in the past twenty-five years, many have remained stationary, and a considerable number have slowly decreased. . . . . The population of the first group in 1880 was 1,342,782, and that of the second group, 440,303" (pp. 762–63).

"Dividing the towns into [this] more accurate classification, without regard to county lines, the towns which may be considered as having a dense population had a mortality rate of 5.15 per 10,000 annually from typhoid fever, while the remaining towns had a mortality rate of 5.54 per 10,000" (p. 823).

This finding, based as it is upon much smaller areas than the counties, approaches much more nearly the study of purely local conditions which alone is instructive for our present purpose, and should therefore be accorded much greater weight.

Dr. Abbott has later stated that it is among that portion of the population not supplied with public water that "typhoid fever largely occurs."[1]

Finally, Dr. Abbott compared the mortality in towns, with the results obtained by Dr. Derby for 1859–68, finding that the same general relations between urban and rural—manufacturing and agricultural—communities still held good in the later period. There is, however, one important difference, viz.:

"The cities which had specially high death-rates from typhoid fever in the later period (1871–90)—Holyoke, Lowell, Lawrence, Fall River and Chicopee—held an intermediate position in 1859–68; and it was not until after a polluted water-supply had been introduced in Lowell and Lawrence that their death-rate from typhoid fever reached an unusually high figure."

[1] Reference Handbook of the Medical Sciences, 1904, 8, p. 254.

The researches of others which we shall summarize are based upon arbitrary statistical divisions of communities into two crude groups of "urban" and "rural." The next in point of time that we have been able to discover is that of Ernst Bolin, based upon the official statistics of Sweden and published at Stockholm in 1893 as a Doctor's thesis presented to the medical faculty of Upsala University, entitled *Tyfoidfebern i Sverige*. The author gives for *närv-* and *hjärnfeber* taken together (equivalent to typhoid fever) the following figures:

	Death-Rates per 100,000 of Population		
	Towns*	Country Villages†	Whole Kingdom
1876–1880	53	31	34
1881–1885	34	26	27
1886–1890	24	22	23
1876–1890	36	26	28

* The *towns (städer)* are distinguished in the official statistics as a separate group. According to the Census of December 31, 1890, there were at that time 92 *städer* with an aggregate population of 899,698. Of these 10 had populations of less than 1,000; and 21 between 1,000 and 2,000. It is obvious that the proportion of the total population contributed by these towns of less than 2,000 is inconsiderable (about 5 per cent), and *for present purposes the Towns group may be taken to represent places of over 2,000 population.*

† The *country villages (landsbygden)*, though including a few places of over 2,000, *practically represent rural communities of less than that figure*, since the population of the few larger places is relatively very small. "Among the places which are not, in law, recognized as towns, there are a few which have a total population of more than 2,000 souls. Some of these places are suburbs of towns; . . . . others are what are called *köpingar* (boroughs), which, from a legal point of view, are something between town and country and, finally, there are some industrial and business places, of which Malmberget . . . . and Trollhattan, with 7,000 inhabitants each, are about the largest" (*Sweden, Its People and Its Industry*, pub. by order of the Swedish Government, 1904, p. 103). The rural region or *landsbygden* comprised in 1890, 3,885,283 inhabitants, including 20 *köpingar* (boroughs). Of the latter, 12 were under 1,000 and 6 from 1,000 to 2,000; the remaining two having 2,205 and 2,075. Thus it is evident that the rural character—as assumed for small communities—of the country villages group is not seriously altered by the inclusion of the few places of over 2,000 population.

Taking *närvfeber* alone (as the term *hjärnfeber* is subject to inexactness), the figures are as follows (p. 56):

	Death-Rates per 100,000 of Population		
	Towns	Country Villages	Whole Kingdom
1876–1880	52	25	29
1881–1885	32	25	27
1886–1890	23	22	22
1876–1890	34	24	26

The conclusion is reached that:

"Typhoid fever, for the entire period, 1876–90, was more general in the towns than in the country villages" (p. 58).

The same conclusion is reached when the proportion of typhoid deaths to the whole number of deaths from all causes is considered (p. 62).

The division here is at a population of 2,000. How far places in Sweden of less than this number of inhabitants represent typical rural conditions we cannot presume to say. The higher group of "towns" is probably a heterogeneous mixture of urban and rural.

To return to American studies, Dr. F. C. Curtis published in the Bulletin of the New York State Department of Health, October, 1900, a statistical study on the distribution and etiology of typhoid fever in that state. Though it was not the object of his study to draw conclusions as to urban and rural typhoid, data are given of 20 of the largest cities in New York state, and, by contrast, of small rural towns, the communities falling between these two groups being omitted. The following is an abstract of the principal table presented:

AVERAGE DEATH-RATE FROM TYPHOID FEVER, 1890-1899, IN 20 CITIES OF 20,000 AND OVER POPULATION.

Low Prevalence	Rate per 100,000 Population
New York	17.0
Yonkers	16.0
Kingston	20.0
Utica	20.0
Auburn	25.0
Rochester	26.0
Moderate Prevalence	
Oswego	27.0
Buffalo	31.0
Syracuse	35.0
Newburgh	37.0
Excessive Prevalence	
Poughkeepsie	42.0
Amsterdam	52.0
Binghamton	52.0
Troy	55.0
Elmira	55.0
Very High Prevalence	
Watertown	80.0
Niagara Falls	80.0
Albany	86.0
Cohoes	90.0
Schenectady	100.0
Rural towns (800)	23.0

The "rural towns" mentioned at the end of this table appear to be a group of the smallest communities arbitrarily set apart after the cities, villages, and more populous towns are taken out. The group is thus fairly representative of the small agricultural villages and hamlets, as well as of the purely agricultural regions. There is no exact criterion of population, but, to judge from other statistical tables of the Bulletin, very few of the communities are over 2,000, while all places under that figure are included. The indication is that genuinely agricultural or rural conditions characterize this group, which has a typhoid death-rate below all but four of the large cities. Dr. Curtis comments on the table as follows:

"Some zymotic diseases belong especially to the cities; evidently typhoid fever does not of necessity. The maritime district is almost wholly urban and its annual death-rate of 17.0 and ratio of 0 86 per cent of total mortality is the lowest; and including more than half the entire population reduces the state death-rate to 24.0, which without it would be 31.0. Neither is it on the average a disease of high mortality in rural towns, for they have a constant mortality of from 22.0 to 25.0 per 100,000 population, and districts with the smallest so-called urban population approach nearest to the rural rate" (*Ann. Rpt. N.Y. State Bd. of Health*, New York, 1900, pp. 429–30; also in Bulletin for October, 1900).

The comparison here made between large cities and small "rural towns" we believe to be valuable, so far as it goes. It is to be noted that the indication of these statistics that the "rural towns" are comparatively low in typhoid is the reverse of the conclusions arrived at by Drs. Derby and Abbott in Massachusetts.

The following extracts indicate the statistical analyses—based upon a division of communities into "urban" and "rural," above and below the 8,000 population mark respectively—published by the U.S. Census since 1890. The misleading character of this mode of division, which will be discussed in section 3, must be borne in mind.

"Typhoid fever was slightly higher in 1900 in the rural districts (25.5) than in the cities (25.3), which is the reverse of the case in 1890, when the rate in the cities was 39, and that in the rural districts 31.4" (*Census of 1900*, 3, p. cxlv).

	Number of Deaths from Typhoid Fever per 100,000 of Population					
	Annual Average	1900 to 1904				
		1900	1901	1902	1903	1904
Cities in registration states.....	25.8	28.5	26.5	25.9	24.6	24.0
Rural part of registration states	27.8	34.6	28.8	27.0	24.7	23.8

(*Mortality*, 1900 to 1904, excerpted from table on p. xxi.)

"Comparing the rural and urban districts of the registration states it will be seen that the mortality from typhoid fever was greater in the rural districts than in the cities in each year except 1904, in which it was very slightly greater in the cities" (*ibid.*, p. xxii).

In the *U.S. Mortality Statistics* for 1907 the typhoid fever rates per 100,000 for 1906 and 1907 are given as follows:

	1906	1907
Cities in registration states	34.2	31.7
Rural part of registration states	28.6	26.0

Among the most detailed studies on rural typhoid which we have found is that contained in a paper by Dr. John S. Fulton, published in the Report of the State Board of Health of Maryland for 1903, and entitled "Typhoid Fever: Some Unconsidered Hindrances in Its Prophylaxis." This research is probably the most thorough and authoritative hitherto made. It is, however, unfortunately based almost entirely upon the arbitrary and (for this purpose) misleading classification of the U.S. Census, and the real question of rural typhoid fever, accurately speaking, is hardly touched upon by any portion of the study. We shall briefly summarize that portion of the paper in which the question now at stake is taken up, and from p. 61 may quote the following introduction:

"The belief that typhoid fever is more common in close communities, and that its incidence is heaviest in cities, has its place in the medical mind by a sort of common consent. The grounds of the belief have not been examined, if, indeed, they have been stated. The late Colonel George Waring said in 1878: 'Typhoid is peculiarly a disease of the country rather than of the town.' No similar statement, I believe, is made by any American medical author; but the opposite view, that typhoid is peculiarly a disease of cities, is expressed in the most recent editions of at least four American textbooks on medicines.[1]

"Typhoid fever makes its most striking demonstrations in cities, and the definite influence of sanitary works on its prevalence has made the typhoid rate the accepted index of municipal hygiene. Typhoid fever has, nevertheless, at the present time and in this country a heavier incidence on small communities and rural districts, and probably this has been the case for a long time; for sanitary works have neither such excellence nor so wide employment in American cities as to give urban populations in general good defense against typhoid. On the contrary, the conditions of American cities with respect to water supply and disposal of waste might be expected to produce a constantly higher typhoid mortality than is experienced under rural conditions."

[1] We have been unable, after a careful search through the textbooks of Osler (1901), J. C. Wilson (1896), H. M. Lyman (1892), William Gilman Thompson (1902), and others, to verify this statement.

The following fields for statistical evidence, based upon the Census division into "urban" and "rural," are then considered in detail: registration area, "grand groups" of the Census, and five "grand groups." The principal result of the study is summarized in the table quoted below and prefaced as follows:

"Some doubt may be admitted as to whether these grand divisions can fairly be compared. If the United States be divided into state groups on the basis of the distribution of the population between town and country, we can eliminate to a great extent the influence of latitude and longitude."

	Average Percentage of Rural Population	Average Typhoid Fever Death-Rate per 100,000
Five states in which the urban population was more than 60 per cent of the total	30	25
Six states in which the urban population was between 40 per cent and 60 per cent	49	42
Seven states in which the urban population was between 30 per cent and 40 per cent	67	38
Eight states in which the urban population was between 20 per cent and 30 per cent	75	46
Twelve states in which the urban population was between 10 per cent and 20 per cent	87	62
Twelve states in which the urban population was between 0 and 10 per cent	95	67

The above groups are constituted as follows, in order: *Five states:* Massachusetts, New York, New Jersey, District of Columbia, Rhode Island. *Six states:* Connecticut, Pennsylvania, Delaware, Maryland, Illinois, California. *Seven states:* New Hampshire, Ohio, Michigan, Wisconsin, Missouri, Colorado, Washington. *Eight states:* Maine, Indiana, Minnesota, Louisiana, Montana, Wyoming, Utah, Oregon; *Twelve states:* Vermont, Virginia, South Carolina, Georgia, Florida, Tennessee, Alabama, Texas, Iowa, Nebraska, Kansas, Kentucky. *Twelve states:* West Virginia, North Carolina, North Dakota, South Dakota, Mississippi, Indian Territory, Oklahoma, Arkansas, New Mexico, Arizona, Nevada, Idaho.[1]

"The experience of Maryland supports the general indications of these figures. There is but one large city in the state. The inhabitants of Baltimore are half of the total population of the state. There are but two other towns having as many as 8,000 inhabitants. It may be fairly said that the population of the state is equally

[1] In this part of the study on p. 63 are several misprints or errors. In one case 12 states are mentioned with the names of only 8; in another, 12 states are mentioned with the names of only 11. Several of the figures given do not agree with those given in the statistical tables printed at the close of the paper.

divided between rural and urban conditions. The ratio of typhoid mortality is 1 to 2.5 in favor of Baltimore, a poorly sewered city, supplied with unfiltered water from an extensive and unprotected watershed."

The study closes with a tabulation of the cities of the United States arranged in groups according to population, showing that the typhoid mortality rises somewhat as the populations diminish. But the smallest group given has an average population of over 12,000, so that rural communities are not concerned. A group of almost eight million people remaining over and above the cities, and called 95 per cent rural, shows a higher mortality than any of the city groups.

Dr. Fulton concludes:

"The infection is more heavily seeded in small communities, and the propagation of the disease is in general from the country to the town, rather than from town to country. The explanation of this fact is probably found in the greater care given to the disposal of human excrement as communities grow more populous" (p. 67).

As evidence of the strong persistence of the rural typhoid theory as above elaborated, it is interesting to note that a recent authority, George C. Whipple, cites Dr. Fulton's study with the remarks:

"Contrary to what many people suppose, typhoid fever is more largely a rural disease than an urban disease, meaning by rural small communities in distinction from large communities" (*Typhoid Fever*, New York, 1908, p. 112).

"The autumnal increase of typhoid fever in cities is sometimes referred to as 'vacation typhoid,' the idea being that it is due to patients returning sick from the country. This theory is based on the fact that typhoid fever is at present chiefly a rural disease, and so far as this goes it is correct" (*ibid.*, p. 127).

Mr. Whipple thus concurs in Dr. Fulton's conclusion so far as *distribution* of typhoid fever is concerned. In regard to its *propagation* he goes on to say:

"But the notion of 'vacation typhoid' has been very much overworked, and, as a matter of fact, it does not to any very material extent, account for the summer and autumn increase of typhoid fever in the large cities. In Washington it was estimated that during the summer and autumn of 1906, 85 per cent of the cases were contracted within the city, and studies of imported cases in other cities have given similar figures" (*loc. cit.*).

By way of further comparison, the statistics of the Registrars-General of England and Wales, and of Scotland may appropriately be cited at this point. For Scotland we quote the following mortality figures for typhoid fever since 1899:

Districts	Containing Places Having Populations	Enteric Death-Rates per 100,000 Population							
		1900	1901*	1902	1903	1904	1905	1906	1907
Scotland	...	15	16	12	12	9	8	9	8
Principal town districts	More than 30,000	14	18	11	14	10	7	8	7
Large town districts	10,000 to 30,000	19	17	18	15	11	16	17	15
Small town districts	2,000 to 10,000	17	18	11	11	8	7	7	6
Mainland rural districts	Under 2,000	12	11	11	9	7	5	6	5
Insular rural districts	Under 2,000	10	12	16	11	10	8	11	8

* Census year.

Populations of groups, estimated for 1907, were approximately as follows: principal town districts, 2,178,000; large town, 603,000; small town, 964,000; mainland rural, 919,000; insular rural, 113,000.

The division into "town" and "rural" is made at 2,000 population; and here, as for Sweden, we cannot venture to say whether communities below that figure probably fairly represent rural conditions. The town groups are presumably a mixture of rural and urban, with the latter predominating.

For England and Wales the figures are less detailed, but represent large populations. The following are typhoid fever death-rates per 100,000 of population (corrected for age and sex distribution of population):

England and Wales	Average 1898–1902	Average 1903–7	Year 1908
Urban counties	19.3	9.9	8.4
Rural counties	10.4	6.8	5.6

The basis for these figures is indicated by the following from the report of the Registrar-General for 1901:

"On examining the local distribution of enteric fever it is found that, generally speaking, its mortality varies as the area affected contains either a large or a small proportion of urban population. For this reason a selection from among the counties of England and Wales has been made so as to show (*a*) in one list certain counties that are chiefly urban in character, and contain most of the industrial centers, and (*b*) in another list certain counties that are exclusively rural, or that contain a few small towns which although technically styled urban, nevertheless partake of that character to a slight extent only" (p. xlix).

The total population of these two selected groups comprises about two-thirds of the total population of England and Wales. The division is evidently arbitrary, but by no means blindly so. Inasmuch as a definite attempt is made to distinguish agricultural (i.e., small and segregated) communities from industrial (i.e.,

large and concentrated) communities, the division is much superior to statistical separations made solely on the basis of populations.

English and Scotch statistics thus agree in indicating for the smallest communities, i.e., the segregated agricultural communities, relatively low typhoid mortality. The excessive mortality in the "large town" districts of Scotland is, moreover, especially noteworthy.

As a matter of record the following references are also appended:

A. Dr. John H. McCollom, in a study (1893) of typhoid fever in Boston, by wards, attributed a slight increase in the frequency in Ward 4, a part of Charlestown, in all probability to "the fact that in this locality the majority of the population leave the city for a greater or less length of time during the summer months, and contract typhoid fever in the country."[1]

B. "In England and Wales . . . . [typhoid fever] destroys more lives in proportion to population in towns than in the country. . . . . [In the United States] it is more prevalent in country districts than in cities, and, as Fulton has shown, the propagation is largely from the country to the town. . . . . In Germany the larger cities have comparatively little typhoid fever. . . . ."[2]

C. In a review of an investigation of typhoid fever at Ipswich, England, by Dr. A. M. N. Pringle, medical officer of health, who ascribed a diminution of typhoid fever in that town to the abolition of the "midden" system in favor of sewerage—a writer in the *Boston Transcript* for October 13, 1909, says:

"There is an important lesson for this country in this investigation, since it shows in Ipswich the typhoid conditions under the same kind of sanitary arrangements that obtain in the rural districts here. The autumnal rise in typhoid is so well marked here and its underlying reason is so clearly understood that it has been termed 'vacation typhoid.' It is a feature in northern American cities, and Dr. Pringle's article points directly to what is without doubt the most constant maintaining factor in the case."

D. "There is reason to believe that the term 'vacation typhoid' which has come into use is far from being without meaning."[3]

E. At the New York State Conference of Health Officers held at Rochester, November, 1909, U.S. Surgeon L. L. Lumsden stated:

"In the United States the rate of prevalence of typhoid fever is higher in the rural sections than in the cities and a city, surrounded by and obtaining its food supplies from a section of the country in which the typhoid rate is high, will have a high typhoid rate."[4]

F. A good example of current newspaper opinion is the following which appeared in an editorial in the *Boston Globe*, June 28, 1911:

"Typhoid and other fevers afflict humanity most immediately after summer vacations and are due generally to the insanitary conditions which have surrounded those who have been seeking pleasure and health away from home."

[1] *Boston Med. and Surg. Jour.*, 1893, 129, p. 296.

[2] William Osler, *Practice of Medicine*, 1910, p. 58.

[3] *Bull. N.Y. State Dept. of Health*, 1909, p. 248.

[4] *Ibid.*, 1909, p. 266.

The quotations given demonstrate without need of further comment the confusion of opinion and the obscurity in which the whole subject stands. When authorities differ, some affirming and others denying a widely prevalent opinion that typhoid fever is a "rural" disease, and is spread chiefly from country to city, a careful investigation is evidently required to settle the question by determining the facts.

3. DISCUSSION AND DEFINITION OF THE TERMS "URBAN" AND "RURAL."

The terms "urban" and "rural" are often used in a vague and general sense while at other times an apparent precision is introduced by arbitrary statistical definition. Thus the U.S. Census division of communities into urban and rural above and below the 8,000 population mark, which has been taken as a basis for study by Fulton and others, is a purely arbitrary line of demarkation and obviously not especially preferable to some other and perhaps lower figure.[1]

Nevertheless, as a basis for sanitary studies, it is necessary to bring together, if possible, and to compare, populations living under either closely corresponding or else strongly contrasting sanitary conditions; and this it is by no means easy to do. There are, for example, great differences in many respects in living conditions and therefore in sanitary conditions between (1) isolated farmhouses, (2) country villages, and (3) the somewhat larger and often compact communities which frequently cluster about a mill or a factory employing a hundred or several hundreds of operatives. Yet hundreds of all three of these and of many other classes are included in the "rural" group of the U.S. Census among communities having fewer than 8,000 inhabitants. Obviously, typhoid fever might prevail in group 3 and at the same time be very scarce in group 1; and yet it is often spoken of as "a rural disease" and ought therefore to be more abundant in the sparsely settled country than in the factory village of denser population.

[1] Since the above was written the United States Census (1910) has set the definitive figure at 2,500, which appears preferable to the old practice. We have, however, thought it wise to let our original discussion stand unaltered; first, because the Census division at 8,000 has been generally accepted as a basis by Fulton and others; and secondly, because we have endeavored to develop the general principle that *any* such arbitrary division of population, unless taken in the roughest sense, is misleading.

The sanitary conditions in a community having one, two, or three thousand inhabitants, and technically called "rural," may and often do more closely resemble those of the city than they do those of the country farmhouse or the sparsely settled hamlet. Such places may, for example, have public water-supplies, and sewerage systems, and if built closely around mills or factories, may possess typical city congestion and tenement-house crowding. Other communities, on the other hand, which under the Census division are also called "rural" may be in fact mill towns or factory towns which have gone beyond the stage of country villages but have not arrived at what are essentially city conditions. This state of development means that most of the dangerous conditions of the city have arrived, while public water-supply, sewerage, and adequate sanitary supervision have not yet been secured. "The state of change" has been called "the state of danger," and the dictum certainly applies in community life. Congestion is characteristically a city evil, but it is no less an evil when connected with the overgrowth of small mill or factory communities where it may exist in as great or even greater degree than in larger centers of population. Such communities, we repeat, are often denominated "rural" under any arbitrary classification which takes no account of the actual characteristics and distribution of housing, crowding, and other *sanitary* conditions of the various communities in question.

The definition used by the Census is, moreover, never exact, as the terms "urban" and "rural" are used without qualification. The inexperienced reader will often suppose these arbitrary statistical terms to be used in a true sense and will be misled as to the phenomena involved, while even the experienced reader may take the distinction to be the trustworthy result of careful analysis of conditions. Thus, while convenient for certain purposes, the terms may lead to erroneous confidence in a meaning which does not exist.

Other studies, e.g., those of the New York State Board of Health (see sec. 2) make no exact distinction, but arbitrarily separate out two groups, as by the criterion of incorporation or general order of magnitude of population. In the studies of Bolin, in Sweden, and of the Registrar-General of Scotland, the division

into rural and urban is equally arbitrary, in the former case by political, in the latter by sanitary, districts; although by special inquiry we have found that the lines in both these two cases are drawn at about 2,000 population.

In the study by Dr. S. W. Abbott, published in the Report of the Massachusetts State Board of Health for 1891, a different method is used, various degrees of density of population by area being used as a basis.

In no case, however, so far as we have been able to discover, has any detailed analytical description of the nature of the "rural" and "urban" populations studied been given,[1] a fact to which much vague thinking on this question may be attributed. Nor has any classification by degree of rural conditions, into more than two crude classes, been made. Rigid definitions of the terms must, in view of the complex character of the conditions, be untrustworthy if not misleading, yet it is nevertheless essential that the general conditions connoted by them be kept in mind.

The term "rural" (derived from the Latin *rus*, country) signifies strictly "of or pertaining to the country as distinguished from a city or town." A secondary meaning at once suggested by the primary one is "agricultural"—the first describing the place, the second the occupation of the inhabitants. It becomes necessary, therefore, to rule out from any group of rural communities all having essentially "city" conditions. The chief of these conditions, from the epidemiological standpoint, are: (1) the congestion characteristic of centers of population; and (2) systems for public convenience, such as public water-supplies, public sewerage systems, public milk-supplies, etc. City life is a state of concentration and even more or less congestion; country life of isolation or segregation. Consequently a purely rural area is one in which the dwellings are more or less widely separated and, instead of being dependent on the systems of public convenience mentioned as characteristic of the city, form in that respect wholly independent and disconnected units. Such a condition, strictly speaking, rarely exists, for everywhere little clusters of dwellings, or "hamlets,"

[1] A partial exception to this statement is found in the *Mass. State Census*, 1905, I, p. xxxii, where dissatisfaction with the U.S. Census division is expressed and a subdivision into rural (under 5,000), semi-urban (5,000–8,000), and urban (over 8,000) is made.

tend to spring up. It is, however, approximated in all really country life for even where there is community in certain sanitary conditions, such as, for example, a common source of provisions (many of which are, however, eaten only after cooking), or through a common school or a common church, the great sanitary factors, such as water-supply, milk-supply, and sewerage, remain practically separate, each dwelling having its own well, its own dairy, and its own privy or cesspool. Personal contact is also reduced to its lowest terms, and is largely confined to families. It is, then, between these two opposing conditions of *concentration* or *segregation* of homes or families that the study of urban vs. rural typhoid must be made; and it is only in the sense of *segregation in the country* that the term "rural" should be used for sanitary studies.

In given instances the conditions are usually complex. Congestion typical of city life may exist in one corner of a community otherwise rural in character, e.g., in a small factory village which though crowded lacks the city characteristics of common water-supply, sewerage, and milk-supplies. On the other hand, the suburbs of a city often have common water-supplies, milk-supplies, and sewerage, and yet approach rural conditions as to segregation of dwellings, absence of congestion, and of personal contact. In no instance can hard and fast lines be drawn, but the principle separating the two groups holds good. For statistical purposes it might be wished that the sanitary phenomena of large numbers of segregated farms could be studied and contrasted with those of typical cities, but practically, it is convenient to take separate towns (in New England), as the smallest units statistically available, and group them together for study according to their population.

To recapitulate, it is plain that any arbitrary division of population into "urban" and "rural," statistical or otherwise, which leaves out of account the facts we have mentioned is misleading. To conduce to a clear understanding of the conditions to be contrasted, we have framed the following characterizations of urban and of rural communities. Hereinafter we shall use the terms solely in these senses, except as otherwise indicated:

A rural community is one characterized by sparsity of population, with segregation of a few families upon a large area; it is accord-

ingly one in which the chief sanitary factors of water-supply, milk-supply, and sewerage are entirely separate for each household, and in which personal contact and association between the members of different families are reduced to their lowest terms.

An urban community is one characterized by density of population with concentration of many families upon a small area; it is therefore one in which some at least of the chief sanitary factors are common to many households, and in which much personal contact and association exist between the members of different families.

4. EVIDENCE DERIVED FROM THE VITAL STATISTICS OF THE STATE OF MASSACHUSETTS.

For the purpose of answering the apparently simple question which forms the subject of this paper, we have made a careful analytical and statistical study calculated to disclose the relative amounts of urban and rural typhoid fever, first, and especially, in Massachusetts, and afterward in the adjoining states of Connecticut and New Hampshire. Moreover, since urban and rural conditions invariably merge each into the other, and since the main object of our study has been to determine how the relative proportions of each affect the prevalence of typhoid fever, any merely rough or arbitrary division into these two classes is not enough. Our studies deal instead with a considerable number of *community groups* and have been designed to be in this respect much more searching, than any, as far as we know, hitherto made.

For several reasons we have chosen for intensive study the mortality statistics of the state of Massachusetts: first, because in Massachusetts we have statistics of approved accuracy covering a period of many years; in the second place, because Massachusetts either has been or now is the home of all three of the authors, and is therefore personally known to them in much detail; in the third place, both agriculture and manufacturing have long been and still are of great importance in Massachusetts, so that both the segregative influences of American agricultural life and the concentrative influences of industrial towns are here at work, side by side; fourthly, we have in this state the important advantage of the possibility of comparing the results of our own studies with the

earlier findings, already referred to, of Dr. Derby and Dr. Abbott, the accomplished secretaries of a state board of health highly reputed for its sanitary investigations and its leadership in sanitary subjects. Finally, we have in two states, contiguous to Massachusetts (Connecticut of much the same character as Massachusetts though somewhat less densely populated, and New Hampshire much more sparsely settled than either of the other states) materials for valuable "controls" and comparisons.[1]

As a convenient and suitable statistical unit for our studies we have taken in all three states the New England "town"[2] which is thus defined by the *Century Dictionary:* "In many of the states one of the several subdivisions into which each county is divided, more accurately called, in the New England states and some others, township."

We have already pointed out above that any comparison of urban mortality with rural mortality over large areas is certain, in Massachusetts at least, to be inconclusive, for the reason that all over even the more rural portions of Massachusetts there are scattered spots or small areas to which the term "urban" ought in all fairness to be applied. These may be either old and settled communities which may or may not have supplied themselves with all modern conveniences such as public water-supply and sewerage systems, boards of health, and even garbage and refuse disposal; or they may be factory villages composed chiefly of tenement houses clustered about some busy mill or workshop but as yet destitute of all sanitary appliances and therefore from a sanitary standpoint perhaps actually worse than the neglected alleys of a crowded city. Obviously the blending of the mortality statistics of such spots as these with those of much larger and sparsely settled farming or forest areas into one group to which the term "rural" is arbitrarily applied, and any comparison of the net result with data drawn from places really urban, can give no true idea as to the relative prevalence of disease under strictly rural and

[1] According to the *Census of 1900*, Massachusetts stood second in the Union in order of density of population, with 349 persons per square mile; Connecticut fourth, with 188; and New Hampshire 15th, with 46.

[2] Certain of these are more exactly described as "cities," so that where towns and cities are grouped together we shall often employ the term "communities."

urban conditions. What we want is rather a comparison of the mortality of sparsely settled farming or forest areas with the mortality of closely crowded urban districts, and this, fortunately, we can approximately obtain in the New England states, divided as these are into towns (townships) usually of very moderate area and hence often entirely rural. Counties, on the other hand, in New England, as elsewhere, generally comprise much larger areas and include community groups of all sorts among which are often large cities.

The New England "town" as a community group may, to be sure, be large or small either in area or in population or in both, but as a rule the statements just made hold good and there are in every New England state many small towns which are in every respect strictly rural. Some, on the other hand, have, perhaps in one corner of the town, a little manufacturing settlement gathered about a mill or factory and it is true that:

> "If the town is large the presumption is that it contains a considerable urban population, while if it is small it is probably all or nearly all rural" (*Mass. State Census*, 1905, I, p. xxxi).

The presumption here well set forth becomes a matter of reasonable certainty when the towns are viewed, as we shall view them, in considerable numbers.

For the purposes of our investigations of the mortality statistics of Massachusetts towns, we have used the following methods:

First, we have consulted the state registration reports for the years 1890–1907 inclusive and have computed from the data therein contained the annual death-rates from typhoid fever for the entire 18 years for every town in Massachusetts. The towns have then been arranged in 13 groups, according to their population, and the death-rates of the several *groups* from typhoid fever have also been computed. The results of both procedures are laid down in the following table (p. 163).

On Fig. 1 we have undertaken to show for the communities there grouped three sets of facts: first, by the *base line*, the average aggregate population for each group during the period under consideration; second, upon these base lines we have placed as *verticals* the average typhoid death-rates for the same period. The

TYPHOID FEVER DEATH-RATES (PER 100,000 OF POPULATION) IN MASSACHUSETTS COMMUNITIES GROUPED ACCORDING TO POPULATION. COMPUTED FROM THE MASSACHUSETTS STATE REGISTRATION REPORTS, 1890–1907, INCLUSIVE.

Population Group	Number of Communities	Average Population 1890–1907	Rates by Years																		
			1890*	1891	1892	1893	1894	1895*	1896	1897	1898	1899	1900*	1901	1902	1903	1904	1905*	1906	1907	Average Rate, 18 Years
Less than 500 persons	36	12,300	31	19	54	35	17	38	31	0	31	23	15	0	14	0	24	0	16	0	19.3
500 to 1,000	57	44,600	32	28	17	23	42	33	17	34	25	9	12	5	5	12	10	15	17	2	18.8
1,000 to 1,500	47	57,600	25	28	36	26	21	14	19	19	20	25	17	10	14	12	13	23	14	10	19.3
1,500 to 2,000	38	66,500	30	40	24	20	21	15	30	17	14	17	18	20	6	9	11	14	21	16	19.1
2,000 to 3,000	42	101,400	40	36	33	27	32	23	20	22	24	18	15	11	16	16	11	11	10	10	20.7
3,000 to 4,000	24	79,000	28	29	15	29	20	25	26	9	27	21	18	22	27	14	10	11	11	3	19.3
4,000 to 5,000	22	99,000	26	33	23	23	34	25	29	12	11	6	12	17	15	17	14	15	8	5	18.1
5,000 to 8,000	34	212,000	23	30	47	36	33	27	21	19	19	18	18	18	15	13	11	15	11	5	21.1
8,000 to 10,000	10	90,560	40	32	30	18	25	22	22	20	34	19	18	13	15	10	16	12	20	14	21.2
10,000 to 15,000	16	197,000	29	33	39	32	31	33	27	22	26	22	20	20	20	13	14	19	15	13	23.8
15,000 to 25,000	7	149,900	25	27	28	35	32	36	28	21	25	28	25	18	22	22	20	14	14	19	24.5
25,000 to Worcester (110,000), incl.	20	1,042,000	53	43	43	35	33	25	30	24	22	22	25	20	18	21	15	18	15	16	26.5
Boston	1	528,700	33	33	30	28	28	32	33	31	33	28	25	24	24	20	21	20	20	10	26.3
The state	354	2,680,560	37	36	35	31	31	27	28	23	25	22	22	20	19	18	16	17	16	13	24.3

* Census years. Population for intervening years estimated by method of U.S. Census.

area of the resultant rectangle represents the actual number of deaths in each group annually. In this way we have plotted both the death-rates and the absolute number of deaths from typhoid fever during the period under consideration in Massachusetts communities of various sizes.

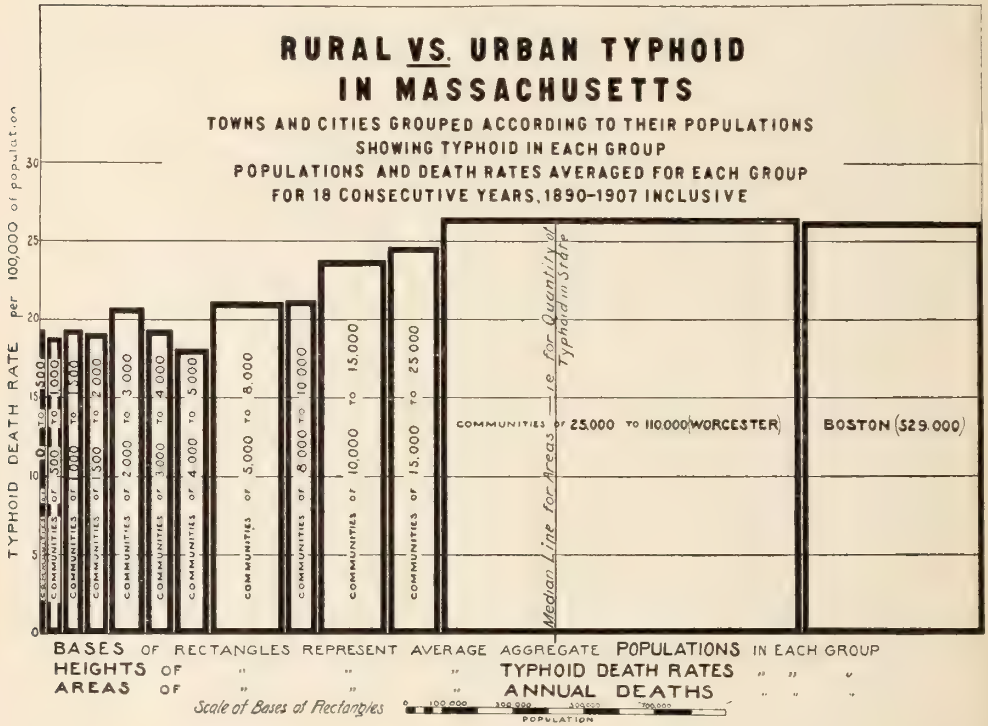


Fig. 1.

It will be observed that the plate shows a fairly regular increase in the death-rates as we proceed from communities of small to those of larger size; and if we consider the areas, that is the absolute quantity or number of deaths, the great balance in favor of the smaller communities and against the larger is very striking. As some further gauge of the distribution of the typhoid deaths, we have indicated in the diagram a line which may be called the *median line* determined by the fact that it divides the total area, that is, the total number of deaths, into two equal parts. In this way a

TYPHOID FEVER MORTALITY IN MASSACHUSETTS COMMUNITIES GROUPED ACCORDING TO POPULATION. DEATH-RATES PER 100,000 OF POPULATION. SIX CENSUS (U.S. OR STATE) YEARS ONLY. COMPILED AND COMPUTED FROM THE STATE REGISTRATION REPORTS.

Population Group	1880				1885				1890			
	Number of Communities	Sum of Component Populations	Sum of Typhoid Deaths	Typhoid Death-Rate	Number of Communities	Sum of Component Populations	Sum of Typhoid Deaths	Typhoid Death-Rate	Number of Communities	Sum of Component Populations	Sum of Typhoid Deaths	Typhoid Death-Rate
Less than 500 persons	22	7,829	3	38	23	7,981	7	88	28	9,772	3	31
500 to 1,000	61	46,474	19	41	72	55,188	19	34	67	52,233	17	33
1,000 to 2,000	92	132,469	66	50	83	122,096	48	39	84	122,798	34	28
2,000 to 3,000	53	127,201	91	72	54	133,250	57	43	48	119,231	45	38
3,000 to 4,000	32	113,509	58	51	29	103,728	41	40	22	75,619	31	41
4,000 to 5,000	28	124,359	77	62	24	106,606	35	33	30	134,154	32	24
5,000 to 6,000	10	54,408	38	70	9	49,816	16	32	7	37,036	7	19
6,000 to 7,000	4	25,530	7	27	8	43,763	22	50	9	57,344	12	21
7,000 to 8,000	6	44,274	15	34	4	29,100	9	31	9	65,825	18	27
8,000 to 9,000	4	33,021	13	39	6	59,547	28	47	5	42,672	14	33
9,000 to 10,000	2	18,338	16	87	5	46,297	9	19	5	47,173	22	47
10,000 to 11,000	5	52,389	16	31	2	21,681	10	46	5	52,462	15	29
11,000 to 12,000	3	34,109	18	53	2	23,266	11	47	2	22,147	2	9
12,000 to 13,000	3	36,618	17	46	3	37,581	17	45	1	12,103	0	0
13,000 to 14,000	3	40,510	22	54	2	26,891	7	26	3	41,251	15	36
14,000 to 15,000	0	......	..	..	2	29,075	5	17	2	29,040	14	48
15,000 to 20,000	3	54,796	15	27	3	51,541	17	33	4	68,785	24	35
20,000 to 25,000	4	89,843	46	51	4	87,955	32	36	4	94,098	17	18
25,000 to 50,000	6	214,134	128	60	8	267,362	111	42	10	344,219	157	46
50,000 to 100,000	3	170,435	63	37	4	249,024	116	47	5	362,504	208	57
100,000 or over	1	362,839	151	42	1	390,393	148	38	1	448,477	146	33
The state	345	1,783,085	879	49.3	348	1,942,141	765	39.4	351	2,238,943	833	37.2

TYPHOID FEVER MORTALITY IN MASSACHUSETTS COMMUNITIES IN (SIX) CENSUS YEARS—*Continued.*

Population Group	1895				1900				1905			
	Number of Communities	Sum of Component Populations	Sum of Typhoid Deaths	Typhoid Death-Rate	Number of Communities	Sum of Component Populations	Sum of Typhoid Deaths	Typhoid Death-Rate	Number of Communities	Sum of Component Populations	Sum of Typhoid Deaths	Typhoid Death-Rate
Less than 500 persons	36	13,287	5	38	37	13,383	2	15	36	12,513	0	0
500 to 1,000	59	45,804	15	33	53	41,442	5	12	54	40,537	6	15
1,000 to 2,000	86	124,719	18	14	89	129,991	23	18	85	123,870	23	19
2,000 to 3,000	41	100,555	23	23	37	88,785	14	16	43	100,993	11	11
3,000 to 4,000	23	76,902	19	25	27	93,751	17	18	18	62,709	7	11
4,000 to 5,000	23	104,395	25	24	17	76,412	9	12	20	88,249	13	15
5,000 to 6,000	15	80,171	16	20	19	102,514	20	20	15	81,638	14	17
6,000 to 7,000	11	69,668	19	27	7	44,836	8	18	13	84,643	13	15
7,000 to 8,000	10	76,315	26	34	11	81,609	14	17	10	73,437	9	12
8,000 to 9,000	6	50,796	13	26	4	34,212	3	9	5	42,453	5	12
9,000 to 10,000	4	37,891	7	18	5	47,549	15	32	4	37,496	5	13
10,000 to 11,000	2	21,170	10	47	3	31,233	1	32	2	20,286	3	15
11,000 to 12,000	5	58,385	16	27	6	67,994	19	28	5	56,510	9	16
12,000 to 13,000	0	......	..	..	3	37,664	5	13	5	61,964	13	21
13,000 to 14,000	0	......	..	..	4	54,404	5	9	3	39,814	10	25
14,000 to 15,000	4	58,181	20	34	2	28,732	10	35	6	86,252	16	19
15,000 to 20,000	5	87,038	37	43	4	75,989	19	25	3	54,866	13	24
20,000 to 25,000	3	62,049	17	27	5	117,682	27	23	3	65,777	4	6
25,000 to 50,000	10	308,466	68	22	10	348,917	92	26	14	483,807	94	19
50,000 to 100,000	9	627,471	166	26	7	504,071	120	24	7	556,589	94	17
100,000 or over	1	496,920	160	32	3	784,176	204	26	3	829,277	157	19
The state	353	2,500,183	680	27.2	353	2,805,346	632	22.5	354	3,003,680	519	17.3

certain size of community is found such that the two groups of communities of small size and larger size, respectively, have the same absolute number of deaths. The median line, therefore, indicates where the actual deaths would exert their center of gravity, so to speak; and for Massachusetts, as shown upon Fig. 1, this median line falls in the left-hand part of the "25,000 to Worcester (incl.)" group. Similar subdivision into smaller groups would show exactly at what size of community the median line falls, but the example just given is sufficient for our purpose. Tried by this test, the bulk of the deaths certainly does not rest upon the more rural towns but rather upon the least rural namely, the largest cities. From this demonstration, therefore, we arrive at our first conclusion, which is that *typhoid fever is not chiefly a rural but rather an urban disease.* Obviously, however, this conclusion applies only to what we may call the mass of typhoid, or of typhoid fever microbes or of typhoid fever material, which would naturally be expected if gathered together to be greater in large than in small populations.

Next, in order to eliminate inaccuracies due to estimates of populations for non-censal years, and to carry our group analysis further, we have studied for six Census years 1880, 1885, 1890, 1895, 1900, and 1905 the typhoid fever mortality of the various communities of Massachusetts arranged according to their population into 21 groups. The rate columns in the above table were calculated by computing for each Census year the typhoid fever death-rate for every town in Massachusetts, each town being assigned to the group into which it fell for the particular Census year. The table shows the results of these computations.

We have then averaged the first two Census years against the last four. The results are shown in the following table (p. 168).

The figures given in this last table are plotted for easy reference and futher study upon Fig. 2, in which the method of presentation corresponds with that used in Fig. 1 and in which the same scales are employed for both upper and lower diagram, and Fig. 2, taken together with the table, demonstrates some of the most striking results of our investigation, namely, as follows:

1. In the first place it appears that the total population of the

state increased between the earlier and the later years about 40 per cent. This increase was chiefly among the cities having more than 25,000 population, although it was shared by many communities ranging from 5,000 upward. On the other hand, population in towns having less than 5,000 actually decreased by some 15 per cent. This movement of the population must be carefully kept in mind wherever conclusions are to be drawn.

TYPHOID FEVER DEATH-RATES (PER 100,000 OF POPULATION) IN MASSACHUSETTS COMMUNITIES GROUPED ACCORDING TO POPULATION (CENSUS YEARS).

Communities Having Populations of	Averages for 1880 and 1885		Averages for 1890, 1895, 1900, and 1905	
	Population	Typhoid Death-Rate	Population	Typhoid Death-Rate
Less than 500	7,900	63	12,240	21
500 to 1,000	50,830	38	45,000	23
1,000 to 2,000	127,290	45	125,340	20
2,000 to 3,000	130,230	58	102,390	22
3,000 to 4,000	108,620	46	77,250	24
4,000 to 5,000	115,490	48	100,800	19
5,000 to 6,000	52,110	51	75,340	19
6,000 to 7,000	34,650	39	64,120	20
7,000 to 8,000	36,690	33	74,300	22.5
8,000 to 9,000	46,280	43	42,530	20
9,000 to 10,000	32,320	53	42,530	27.5
10,000 to 11,000	37,030	39	31,290	31
11,000 to 12,000	28,690	50	51,260	20
12,000 to 13,000	37,100	46	27,930	11
13,000 to 14,000	33,700	40	33,870	23
14,000 to 15,000	14,540	17	50,550	34
15,000 to 20,000	53,170	30	71,670	32
20,000 to 25,000	88,900	44	84,900	18.5
25,000 to 50,000	240,750	51	371,350	28
50,000 to 100,000	209,730	42	512,660	31
100,000 to 130,000		..	114,300	27.5
Boston	376,620	40	525,420	27.5
State of Massachusetts	1,862,610	44.35	2,637,040	26.05

2. Inasmuch as the deaths were, on the whole, somewhat less for the various communities during the later years (owing, no doubt, to general sanitary improvements) and while, as we have just said, the population increased during the same period, the typhoid fever death-rates, as indicated by the diagram, declined for the most part conspicuously, between the earlier and the later years.[1]

[1] The question may be raised at this point as to how far this decline in death-rates is attributable to improved methods of diagnosis. It is of course true that with the establishment and growth of hospitals and the general improvement of facilities for medical observation and investigation, accuracy in the diagnosis of typhoid fever has been much increased since 1880. We are not at present, however, especially concerned with the reasons underlying the general decline in death-rates, and we see no reason for suspecting that any quantitative relation of rural and urban typhoid has been much obscured by changes in methods of diagnosis, or that improved diagnosis could have brought about any merely apparent reversal in that relation between 1880 and 1907. We shall return to this subject in a later section.

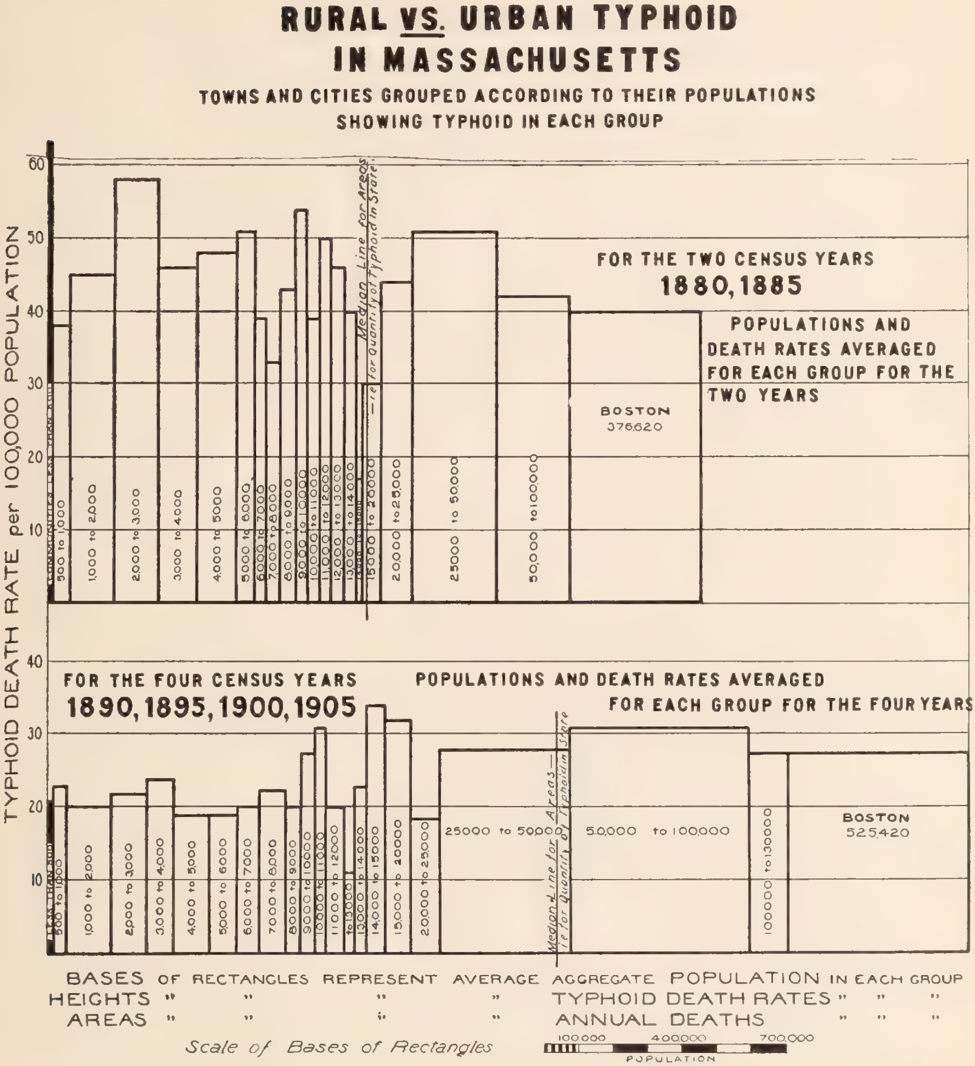

RURAL VS. URBAN TYPHOID
IN MASSACHUSETTS
TOWNS AND CITIES GROUPED ACCORDING TO THEIR POPULATIONS
SHOWING TYPHOID IN EACH GROUP
TYPHOID DEATH RATE per 100,000 POPULATION
FOR THE TWO CENSUS YEARS
1880, 1885
POPULATIONS AND DEATH RATES AVERAGED FOR EACH GROUP FOR THE TWO YEARS
Median Line for Areas — i.e. for Quantity of Typhoid in State.
BOSTON
376620
FOR THE FOUR CENSUS YEARS
1890, 1895, 1900, 1905
POPULATIONS AND DEATH RATES AVERAGED FOR EACH GROUP FOR THE FOUR YEARS
25000 to 50000
50,000 to 100,000
100000 to 130000
BOSTON
525420
BASES OF RECTANGLES REPRESENT AVERAGE AGGREGATE POPULATION IN EACH GROUP
HEIGHTS " " " " TYPHOID DEATH RATES " " "
AREAS " " " " ANNUAL DEATHS " " "
Scale of Bases of Rectangles
100,000 400,000 700,000
POPULATION

Fig. 2.

3. The decline in the amount of typhoid fever as indicated by the decline in death-rates from that disease was considerably greater among the smaller than among the larger communities. This decline is especially noteworthy in the groups having less than 6,000 population.

4. The median line for areas (i.e., for the number of deaths) falls for 1880 and 1885 in the vicinity of populations of 15,000, and for the later years has moved toward a much larger community-size, namely, about 50,000 population.

5. The most important result disclosed by the diagram for our purposes is, however, that in the earlier period the groups of smaller communities showed the highest typhoid fever death-rates, while in the later years this relation is reversed.

The results here obtained are in close accordance with the findings, referred to above, of Dr. S. W. Abbott, who confirmed the earlier study of Dr. Derby, and attributed to the smaller communities previous to 1891 a relatively greater prevalence of typhoid fever. It appears, however, that (as is stated in the last paragraph) an exact reversal of this relation appears to have taken place since the period covered by Dr. Abbott's study, namely, 1871–90. It becomes an interesting question what precisely has led to this singular reversal, which apparently compels us to conclude that whereas before 1890 typhoid fever was in fact a rural disease, since that time it has gradually become an urban disease—a curious circumstance, and one which, if confirmed, justifies both the conclusions of earlier authorities like Drs. Derby and Abbott, and also the suspicion and incredulity which impelled us to undertake the present investigation. To a further consideration of this point we shall return hereafter.

Finally, in order to carry into still greater detail the studies described above and to establish a basis of comparison for the present time with the interesting studies of individual towns made by Drs. Derby and Abbott, we have calculated for the period of 18 years already under consideration, beginning with 1890, the average annual death-rate from typhoid fever for the various communities of the state of Massachusetts. These have then been arranged in the order of their typhoid fever death-rates in the following table:

AVERAGE ANNUAL TYPHOID FEVER DEATH-RATES PER 100,000 OF POPULATION, MASSACHUSETTS COMMUNITIES, 1890–1907, INCLUSIVE.

Community	Average Population 1890 1907	Typhoid Death-Rate
Towns with No Deaths		
Gosnold	150	0
Alford	281	0
Boxborough	318	0
Prescott	370	0
Shutesbury	413	0
Plainfield	418	0
Westhampton	472	0
Wendell	501	0
Halifax	519	0
Rowe	530	0
Plympton	537	0
Windsor	549	0
Oakham	612	0
Richmond	694	0
Princeton	954	0
Millis	1,024	0
Wellfleet	1,051	0
Sandwich	1,570	0
Total population	10,963	
Plainville (1905–7 only. Incorporated 1904)	1,300	0
Westwood (1900–1907 only. Incorporated 1899)	1,124	0
Towns with Rate 0–5		
Nantucket	3,031	1.8
Rehoboth	1,857	3.0
Freetown	1,421	4.0
Pembroke	1,261	4.2
Bedford	1,169	4.4
Total population	8,739	
Towns with Rate 5–10		
Ashfield	988	5.4
Stow	963	5.5
Southwick	991	5.6
Gill	1,020	5.8
Truro	811	6.2
Shrewsbury	1,616	6.7
Lynnfield	822	6.8
Ashby	842	6.9
Holbrook	2,377	6.9
Wellesley	4,938	7.0
Brookline	18,679	7.2
Marion	890	7.3
Granby	780	7.3
Falmouth	3,032	7.7
Hopkinton	2,957	8.0
Georgetown	1,960	8.5
Rutland	1,251	8.6
Walpole	3,379	8.7
Orleans	1,148	9.2
Burlington	593	9.5
West Bridgewater	1,845	9.6
Marshfield	1,761	9.6
New Braintree	523	9.7

Community	Average Population 1890–1907	Typhoid Death-Rate
Sudbury	1,162	9.7
Easthampton	5,551	9.9
Whitman	5,868	9.9
Lincoln	837	9.9
Harvard	1,118	9.9
Bourne	1,616	9.9
Total population	70,318	
Towns and Cities with Rate 10–15		
Holliston	2,649	10.4
Groton	2,138	10.5
Southboro	2,047	10.5
Warwick	577	10.6
Canton	4,625	10.8
Raynham	1,515	10.9
Manchester	2,201	10.9
Lexington	3,849	10.9
Savoy	530	11.0
Enfield	988	11.1
Southampton	1,102	11.1
Westminster	1,425	11.1
Newbury	1,499	11.1
Ashland	1,936	11.1
Hanover	2,119	11.1
Hingham	4,843	11.5
Heath	444	11.7
Conway	1,388	11.7
East Longmeadow	1,368	11.9
Shirley	1,465	11.9
Easton	4,695	12.0
Lancaster	2,316	12.1
Monterey	464	12.2
Mendon	910	12.2
Ayer	2,270	12.2
Paxton	443	12.3
Seekonk	1,593	12.4
Avon	1,663	12.4
Hatfield	1,672	12.4
Hawley	465	12.5
Chatham	1,786	12.5
North Brookfield	3,935	13.0
Milton	6,048	13.0
Yarmouth	1,622	13.1
Florida	419	13.3
Monson	3,827	13.5
Medway	2,827	13.6
Littleton	1,390	13.7
Brewster	849	13.7
Dedham	7,432	13.7
Whately	781	13.8
Bridgewater	5,518	13.9
Bernardston	777	14.0
Hampden	729	14.1
Bolton	789	14.2
Hull	1,449	14.2
Hamilton	1,394	14.3
Belmont	3,453	14.3
Franklin	5,091	14.3
Shelburne	1,529	14.5
Northfield	1,933	14.5
Mansfield	3,906	14.5
Ware	8,044	14.5
Merrimac	2,237	14.6
Barnstable	4,219	14.6
Medfield	2,509	14.7
Marlboro	14,165	14.7

AVERAGE ANNUAL TYPHOID FEVER DEATH-RATES PER 100,000 OF POPULATION, MASSACHUSETTS COMMUNITIES, 1890–1907, INCLUSIVE—*Continued.*

Community	Average Population 1890–1907	Typhoid Death-Rate
Boxford	740	14.8
Mattapoisett	1,105	14.8
Gloucester	26,428	14.9
Fitchburg	28,886	14.9
Total population	201,016	
TOWNS AND CITIES WITH RATE 15–20		
Millbury	4,719	15.0
Winchester	6,854	15.0
Dana	742	15.1
Kingston	1,891	15.1
Stoneham	6,256	15.1
Leominster	11,077	15.1
Sheffield	1,859	15.2
Stoughton	5,457	15.4
Spencer	7,670	15.6
Tyngsboro	709	15.7
Hadley	1,764	15.7
Wayland	2,152	15.8
Winthrop	5,292	15.8
Topsfield	1,045	15.9
Wareham	3,487	16.0
Swampscott	4,149	16.2
South Hadley	4,623	16.2
Saugus	5,109	16.2
Concord	5,251	16.3
Middleboro	6,695	16.3
Peabody	11,501	16.3
West Newbury	1,600	16.4
Rockport	4,673	16.5
Newton	31,375	16.5
Hardwick	3,022	16.7
Revere	9,485	16.7
Swanzea	1,642	16.8
Peru	283	17.0
Sterling	1,299	17.0
Essex	1,688	17.0
Duxbury	1,994	17.0
Pepperell	3,381	17.0
East Bridgewater	3,014	17.0
Montague	6,412	17.0
Amherst	4,963	17.2
Andover	6,465	17.2
Foxboro	3,217	17.3
Fairhaven	3,709	17.3
Danvers	8,422	17.5
Auburn	1,689	17.6
Arlington	7,849	17.6
Goshen	298	17.7
Brimfield	974	17.8
Bellingham	1,547	17.8
Westboro	5,381	17.8
Melrose	12,291	17.8
West Boylston	2,404	18.0
Deerfield	2,458	18.0
Templeton	3,338	18.0
Barre	2,283	18.1
Medford	16,450	18.1
Buckland	1,516	18.2
Reading	4,977	18.2
Mashpee	312	18.3
Norfolk	963	18.3
Middleton	917	18.5
Berlin	922	18.5
Cambridge	87,050	18.5
Worcester	110,400	18.5
North Andover	4,087	18.7
Monroe	288	18.8
Wenham	886	18.8
North Reading	912	18.9
Eastham	525	19.0
West Brookfield	1,473	19.0
Chesterfield	593	19.1
Weston	1,851	19.1
Northboro	2,001	19.2
Upton	1,997	19.3
Watertown	9,215	19.3
Framingham	10,537	19.6
Sherborn	1,422	19.7
Hinsdale	1,581	19.7
Longmeadow	1,144	19.8
Brookfield	2,983	19.9
Westford	2,426	19.9
Hyde Park	12,723	19.9
Total population	505,505	
TOWNS AND CITIES WITH RATE 20–25		
Cohasset	2,602	20.0
Egremont	790	20.1
Abington	4,557	20.[illegible]
Montgomery	268	20.3
North Attleboro	7,164	20.4
Sunderland	760	20.5
Braintree	5,885	20.7
Plymouth	9,180	20.7
Lynn	67,336	20.7
Southbridge	9,439	20.8
Everett	22,011	20.8
Warren	4,432	20.9
West Springfield	6,805	20.9
Athol	7,062	21.0
Billerica	2,644	21.1
Wakefield	8,935	21.1
Charlemont	1,027	21.2
Dartmouth	3,460	21.2
Norwell	1,567	21.3
Hubbardston	1,263	21.4
Stockbridge	2,078	21.6
Chelmsford	3,624	21.6
Somerville	57,790	21.6
Sharon	1,874	21.7
Taunton	29,061	21.7
Malden	32,050	21.7
Dudley	3,436	21.8
Great Barrington	5,335	21.8
Palmer	7,315	21.8
Brockton	38,424	21.8
Ashburnham	1,900	21.9
Northampton	17,915	22.0
Acton	2,016	22.1
Leicester	3,317	22.1
Clarksburg	1,009	22.2
Greenfield	7,391	22.2
Chelsea	33,260	22.3
Carlisle	494	22.4
Erving	1,001	22.4
Lunenburg	1,252	22.4
Gardner	10,335	22.5
Woburn	14,151	22.6
Rockland	5,661	22.8
Hancock	475	22.9
Lenox	2,948	22.9

AVERAGE ANNUAL TYPHOID FEVER DEATH-RATES PER 100,000 OF POPULATION, MASSACHUSETTS COMMUNITIES, 1890–1907, INCLUSIVE—*Continued.*

Community	Average Population 1890–1907	Typhoid Death-Rate
Oxford	2,652	23.0
Marblehead	7,606	23.0
Charlton	1,927	23.1
Orange	5,335	23.1
Quincy	23,101	23.1
Norwood	5,098	23.2
Beverly	13,211	23.2
Huntington	1,440	23.3
Tewksbury	3,631	23.3
Wilmington	1,475	23.4
Attleboro	10,293	23.4
Petersham	927	23.6
Berkeley	932	23.6
Nahant	963	23.6
Holden	2,582	23.6
Weymouth	11,319	23.7
Clinton	12,312	23.7
Waltham	24,832	24.0
Westport	2,759	24.3
Lakeville	919	24.4
Natick	9,516	24.6
Dover	672	24.8
Total population	594,800	
TOWNS AND CITIES WITH RATE 25–30		
Townsend	1,776	25.0
Holyoke	43,850	25.6
Washington	388	25.8
Oak Bluffs	1,089	25.8
New Marlboro	1,271	25.8
Winchendon	5,045	26.0
Hudson	5,523	26.2
Colrain	1,702	26.3
Boston	535,417	26.3
Pittsfield	21,633	26.3
Dunstable	414	26.5
Grafton	5,012	26.6
Milford	10,498	26.7
Maynard	3,851	27.0
Ipswich	4,808	27.0
Haverhill	35,774	27.0
Fall River	95,715	27.2
Webster	7,606	27.4
Middlefield	412	27.5
Holland	230	27.8
Boylston	878	27.9
Norton	1,826	27.9
Uxbridge	3,639	27.9
Williamsburg	1,961	28.0
Dighton	1,889	28.1
Somerset	2,156	28.1
Williamstown	4,682	28.2
Randolph	3,913	28.3
Amesbury	9,494	28.5
Westfield	11,840	28.5
Needham	3,712	28.7
Methuen	6,918	28.7
New Bedford	60,523	28.7
Edgartown	1,166	28.8
Chester	1,374	28.8
Salem	35,204	28.8
Rowley	1,325	29.2
Dracut	2,857	29.2
West Tisbury	453	29.3
Wales	725	29.3
Cummington	758	29.5
Tyringham	368	29.7
Agawam	2,273	29.8
Total population	941,948	
TOWNS AND CITIES WITH RATE 30–40		
Tisbury	1,194	30.1
Northbridge	6,257	30.2
Dennis	2,494	30.7
Salisbury	1,474	30.8
Sturbridge	2,004	31.0
Wrentham	2,324	31.2
Gay Head	165	31.4
Dalton	3,080	31.4
Russell	893	31.9
Wilbraham	1,724	32.1
Otis	528	32.4
Lee	3,865	32.7
Groveland	2,330	33.0
Provincetown	4,433	33.3
Acushnet	1,162	33.6
Chilmark	326	34.8
Phillipston	461	34.9
Pelham	473	34.9
Scituate	2,408	35.0
Royalston	945	35.3
Cheshire	1,246	35.5
Rochester	1,050	36.3
Chicopee	17,869	36.5
Belchertown	2,165	36.9
Hopedale	1,672	37.5
Newburyport	14,471	37.7
Harwich	2,473	38.1
Tolland	313	38.2
Douglas	2,042	38.2
New Salem	601	38.5
Sutton	3,230	38.9
Total population	85,672	
TOWNS AND CITIES WITH RATE 40–50		
Greenwich	493	40.7
Ludlow	3,050	41.7
West Stockbridge	1,232	42.0
Lawrence	59,018	43.4
Granville	995	43.4
Hanson	1,398	43.4
Lanesboro	873	43.6
Lowell	89,100	43.7
North Adams	20,871	46.2
Blandford	825	48.5
Becket	929	48.5
Total population	178,784	
TOWNS WITH RATE OVER 50		
Carver	1,131	50.4
Blackstone	5,904	52.9
Adams	10,317	53.3
Leyden	388	57.6
Sandisfield	732	63.2
Mt. Washington	123	77.4
Leverett	723	84.2
New Ashford	112	90.4
Total population	19,430	

Populations based on U.S. Census for 1890 and 1900, and State Census for 1895 and 1905. Deaths from State Registration Reports.

From these detailed tables another, and general one, may be made enabling us to compare at a glance the typhoid fever death-rates of communities of various sizes (or population) for the 18 years under consideration as follows:

MASSACHUSETTS COMMUNITIES ARRANGED BY POPULATION GROUPS AND TYPHOID FEVER DEATH-RATES (PER 100,000) FOR THE YEARS 1890–1907, INCLUSIVE.

Typhoid Death-Rate at or between	Average Population of Communities, 1890–1907							
	Under 1,000	1,000–2,000	2,000–3,000	3,000–5,000	5,000 10,000	10,000–25,000	25,000–125,000	Over 125,000
0	7,318	3,645	0	0	0	0	0	0
0– 5	0	4,708	0	3,031	0	0	0	0
5–10	9,040	14,497	5,334	11,349	11,419	18,679	0	0
10–15	9,905	28,834	23,313	37,352	32,133	14,165	55,314	0
15–20	9,324	29,002	16,707	55,359	101,813	74,579	229,425	0
20–25	7,200	15,735	20,280	26,457	107,727	159,480	257,921	0
25–30	4,626	15,379	7,286	24,605	39,598	43,971	271,066	535,417
30–40	4,705	9,522	18,240	14,608	6,257	32,340	0	0
40–50	4,115	2,630	0	3,050	0	20,871	141,118	0
Over 50	2,078	1,131	0	0	5,904	10,317	0	0
Totals	58,311	125,083	91,160	175,811	304,851	374,402	961,844	535,417

The data shown upon the foregoing basic or detailed tables are presented graphically upon Fig. 3, where the typhoid fever death-rates of the various communities of Massachusetts as arranged in the tables have been plotted diagrammatically in the same order of succession. The vertical bars represent by their height the population of each community, and in the case of some of the largest towns and cities these bars have had to be broken off in order to bring them within the limits of the diagram. Whenever this has been done the bar has been broken in the middle, the upper half omitted, and the actual population indicated upon the remaining lower half. The authors are personally familiar with many of the towns and cities of Massachusetts, but it would be impossible without unduly lengthening the present paper to take up for consideration the individual communities. We shall therefore merely point out some of the more salient features developed by the diagram.

The observer cannot fail to be struck by the absence from the upper line of the series (which represents communities having the lowest typhoid fever death-rates) of communities of the larger or even medium size. On the contrary, many of the very smallest

here find a place, and, with the single exception of Brookline, there is no community among the first 110 of the series which has a population of more than 8,000 -the dividing line, it will be remembered, of the U.S. Census of 1900 between rural and urban com-

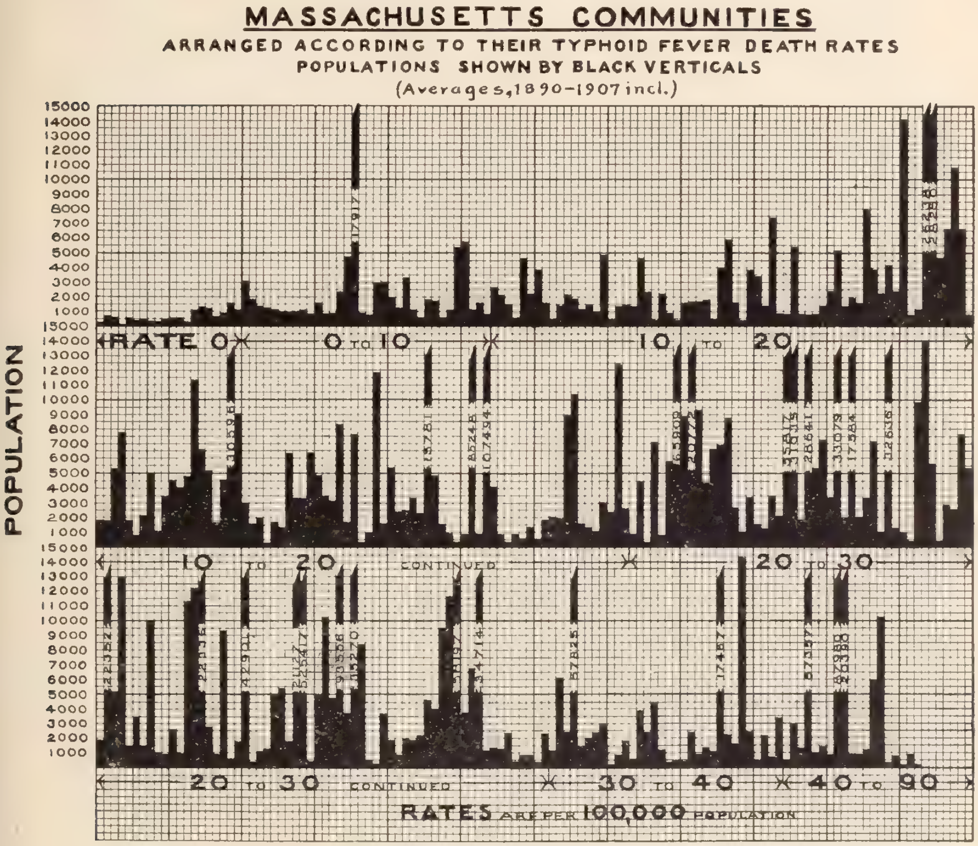


Fig. 3.

munities—while the majority (61) of these, are well under 1,500 population. It is certainly a significant fact that of 20 towns which had no deaths whatever from typhoid fever during the 18 years under consideration, only one had more than 1,500 population.

Passing from the lower death-rates to the higher, towns of medium size and large size become more frequent, although there are scattered among these all the way along a few of the more

strictly rural communities. When we reach the groups having death-rates of 20–30, the number of communities having 15,000 population and upward is remarkable. Rates 30–40 and 40–90 are represented by comparatively few communities of any kind and those of various sizes.

Going more into details we find from rate 0 to rate 10 per 100,000 only one community in 54 with as many as 6,000 population, most of the other 53 falling below 2,000. Low populations continue to be associated with low death-rates well into the next (rate 10 to 20) group but as we advance toward rate 20 per 100,000 a large number of comparatively urban communities appear. These are still more conspicuous as we pass into the region of rate 20 to 30, but become less frequent as we pass the rate 30 line. Beyond this line low populations with high typhoid death-rates are again more abundant, although out of the 51 communities having death-rates of 30 or more per 100,000, nine have 6,000 or more population. Again, out of the 55 communities having the lowest death-rates from typhoid fever 54 had less than 6,000 population, while out of the 51 having the highest death-rate only 42 had less than 6,000.

As to the total population in the rate 0 to rate 10 group we find that this is about 90,000, while that in the group between rate 30 and rate 90 is very much larger. The former is distinctly a rural group; the latter much more urban. In view of this presentation of the subject it is hard to believe that typhoid fever today is, in any true sense of the term, "a rural disease."

We may now bring together the most obvious results of our studies above described upon the prevalence of typhoid fever in Massachusetts communities of various sizes:

Among the twelve groups taken for study for the entire period, 1890–1907, typhoid fever is not found to be either absolutely or relatively to the population a "rural" disease. On the contrary, during this period, typhoid fever has prevailed somewhat more among larger than among smaller communities.

Our second analysis (of 21 groups confined to Census years) confirms this result and in addition brings out the fact that this was not the case prior to 1890, thus confirming the earlier findings

and opinions of Drs. Derby and Abbott and also probably explaining the origin of the belief of today that typhoid fever is a rural disease.

The results of a detailed study of the more than 350 communities into which Massachusetts is divided, confirms and strengthens the foregoing conclusions, by showing that a large number of the smallest and most rural communities are among those having the lowest typhoid fever death-rates, as well as a scarcity among the latter of communities of large size; and, on the other hand, revealing a notable concentration of relatively large communities in the groups having comparatively high death-rates.

We are evidently driven by all our considerations to the conclusion that no matter whether we take as a basis the total amount of typhoid fever material existing in the various communities of the state, or the specific death-rates from that disease, typhoid fever in Massachusetts since 1890 cannot possibly be regarded as "a rural disease." It appears, on the contrary both from our tables and our diagrams, not only that there is very much more typhoid fever in the city than there is in the country, but also that there is more of it there in proportion to the population. Typhoid fever must for the present, therefore, be regarded as an urban rather than a rural disease, at least in Massachusetts.

5. EVIDENCE DERIVED FROM THE VITAL STATISTICS OF THE STATE OF CONNECTICUT.

For comparison with Massachusetts we have next taken Connecticut, a state very similar in general and industrial character of population. The relative distribution of population between communities of smaller and larger sizes is somewhat but not very different in the two states, and here, as in Massachusetts, we have taken the "town" as our statistical community unit. We are fortunate also in having again the reliable vital statistics of a registration state upon which to make our researches.

Corresponding with what we have already done for Massachusetts, we have computed the following death-rates for certain groups of towns in Connecticut:

TYPHOID FEVER DEATH-RATES (PER 100,000 OF POPULATION) IN CONNECTICUT COMMUNITIES GROUPED ACCORDING TO POPULATION. COMPUTED FROM THE CONNECTICUT STATE REGISTRATION REPORTS (1890–1907, INCLUSIVE).

Population Group	Number of Communities	Average Population 1890–1907	Rates by Years																		Average Rate 18 Years
			1890*	1891	1892	1893	1894	1895	1896	1897	1898	1899	1900*	1901	1902	1903	1904	1905	1906	1907	
Less than 500 persons	7	3,040	0	13	31	94	0	32	0	0	0	0	0	0	34	0	0	0	0	0	11.4
500 to 1,000	37	27,100	42	35	21	43	50	25	15	15	7	19	19	11	15	11	19	19	20	16	22.4
1,000 to 1,500	24	31,200	34	51	35	40	28	16	25	19	20	23	24	14	16	13	10	9	12	12	22.4
1,500 to 2,000	18	28,500	50	33	34	27	33	20	11	4	14	9	26	13	14	4	14	21	12	12	19.5
2,000 to 3,000	24	57,100	19	36	27	37	30	21	14	11	15	14	37	15	13	8	13	14	24	20	20.4
3,000 to 4,000	17	65,300	34	29	29	27	19	31	22	11	16	15	21	15	7	7	5	12	12	10	19.6
4,000 to 5,000	7	32,000	54	53	53	40	34	26	19	10	16	22	19	13	25	15	25	28	13	11	26.4
5,000 to 8,000	12	77,800	30	47	39	36	35	23	31	25	10	31	34	14	29	8	15	18	15	24	25.7
8,000 to 10,000	4	35,800	12	52	86	31	29	27	22	15	15	17	62	16	7	20	30	23	20	11	27.5
10,000 to 15,000	6	61,200	47	40	50	26	12	37	22	7	22	21	32	12	15	9	19	25	12	17	23.6
15,000 to 25,000	6	117,000	57	39	36	38	26	46	23	15	16	12	25	25	32	34	13	23	19	33	28.4
25,000 to Bridgeport (67,700)	4	169,000	55	34	36	28	19	20	24	20	22	16	26	25	25	22	17	17	17	20	21.6
Hartford (75,900) and New Haven (104,700)	2	180,600	39	40	48	36	38	42	34	26	10	34	38	74	29	30	21	42	14	25	37.2
State	168	886,840	42	39	40	35	31	31	24	18	22	21	31	30	22	22	17	22	22	20	27.1

* Census years. Population for intervening years estimated by method of U.S. Census.

From the above table we have plotted Fig. 4, in which the method of presentation is the same as that employed in Fig. 1. We must, however, caution the reader that in order to reduce this diagram (as well as the following diagram of New Hampshire data) to the limits of the page, we have used a different base-line scale, and for the same reason, a different vertical scale for death-rates. Hence exact graphical comparisons from one figure to another of this series cannot be made.

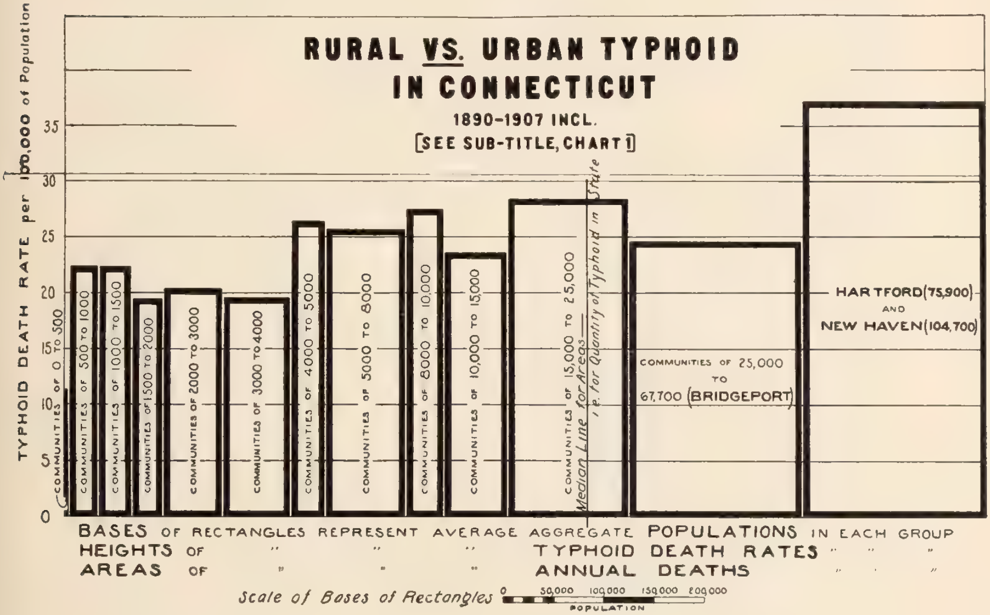


FIG. 4.

Surveying the Connecticut groups, we note the similarity of the distribution of typhoid fever here—whether measured by rates or by total deaths—with that in Massachusetts. The groups of communities of 1,500 to 2,000 and of 3,000 to 4,000 population show the minimum rates of about 19, and from that level the rates range to the group of the largest communities, embracing Hartford and New Haven, which group shows a rate of more than 37. In general also there is a progressive increase of rates from the smallest to the largest communities. If the total deaths, as indicated by

the areas of the rectangles, be taken into account, we find that the bulk of the deaths among communities of over 15,000 population much exceeds that among all the smaller, the median point for the areas (=deaths) falling between 15,000 and 25,000. The total deaths among the smallest communities—say under 3,000—is seen to be but a small proportion of the whole quantity of typhoid mortality.

For Connecticut we have also compiled the average annual death-rates from typhoid fever for each town and city for the 18 years under consideration and the results grouped in the order of such death-rates, from rate zero to rate 50 or more, are shown in detail in the following table:

AVERAGE ANNUAL TYPHOID FEVER DEATH-RATES PER 100,000 OF POPULATION IN CONNECTICUT COMMUNITIES, 1890–1907, INCLUSIVE. COMPILED FROM THE STATE REGISTRATION REPORTS.

Community	Average Population 1890–1907	Average Typhoid Fever Rate 1890–1907
TOWNS WITH NO DEATHS		
Beacon Falls	465	0
Hartland	588	0
Lyme	784	0
Marlboro	361	0
Salem	465	0
Total population	2,663	
TOWNS WITH RATE 0–5		
Preston	2,764	2.2
Stratford	3,450	3.7
Total population	6,214	
TOWNS WITH RATE 5–10		
Monroe	1,036	5.4
Sharon	2,007	5.6
Coventry	1,668	6.4
Willington	889	6.4
Wilton	1,616	6.6
East Haddam	2,502	6.6
Weston	830	6.8
Canton	2,651	8.3
Newtown	3,316	8.4
Lebanon	1,543	8.7
Fairfield	4,395	8.6
Harwinton	1,170	9.9
Total population	23,623	
TOWNS AND CITIES WITH RATE 10–15		
Pomfret	1,777	10.4
Wolcott	573	10.4
East Haven	1,134	10.5
Bethany	522	10.8
Barkhamstead	900	10.8
Easton	967	11.5
Middlefield	868	11.6
Windsor Locks	3,015	11.7
Colchester	2,141	11.9
Essex	2,473	11.9
Bolton	456	12.1
Warren	439	12.2
Goshen	855	12.3
Chester	1,325	12.6
Canterbury	888	12.6
East Windsor	3,118	12.6
Salisbury	3,480	12.8
Union	428	12.9
Plainville	2,159	12.9
Suffield	3,467	12.9
Old Lyme	1,201	13.0
Granby	1,292	13.0
North Branford	817	13.6
Litchfield	3,228	13.8
Darien	2,993	13.9
Norfolk	1,603	13.9
Bridgeport	67,676	14.6
Griswold	3,434	14.7
Saybrook	1,611	14.7
Cheshire	1,960	14.9
Total population	116,820	
TOWNS AND CITIES WITH RATE 15–20		
South Windsor	1,972	15.0
Plainfield	4,785	15.0
Wethersfield	2,582	15.3
Ellington	1,785	15.6
Newington	1,028	15.8
Rocky Hill	1,032	15.9
Guilford	2,784	16.0
Milford	3,787	16.2
Columbia	668	16.6
Middlebury	710	16.7
North Stonington	1,278	17.2
New Milford	4,669	17.4

AVERAGE ANNUAL TYPHOID FEVER DEATH-RATES PER 100,000 OF POPULATION IN CONNECTICUT COMMUNITIES, 1890–1907, INCLUSIVE. COMPILED FROM THE STATE REGISTRATION REPORTS—*Continued.*

Community	Average Population 1890–1907	Average Annual Rate 1890–1907
Norwich	24,400	17.4
Torrington	11,492	17.5
Seymour	3,504	17.5
Bridgewater	644	17.7
Trumbull	1,567	17.8
Colebrook	746	17.9
Berlin	3,321	17.9
Groton	5,898	17.9
Burlington	1,232	18.2
Canaan	843	18.2
Southbury	1,216	18.3
Westbrook	882	18.3
Avon	1,284	18.3
Montville	2,387	18.7
Vernon	8,531	18.8
Woodbridge	864	19.2
Meriden	28,176	19.4
North Haven	2,119	19.9
Total population	126,196	
TOWNS WITH RATE 20–25		
Franklin	551	20.2
Greenwich	11,865	20.2
Prospect	535	20.3
Morris	542	20.3
Woodbury	1,961	20.4
Winchester	7,526	20.7
Sprague	1,304	20.9
Chatham	2,212	21.0
Chaplin	532	21.1
Portland	4,009	21.1
Mansfield	1,839	21.1
New Canaan	2,934	21.1
Thompson	6,311	21.2
Tolland	1,036	21.4
Derby	7,635	21.7
Ashford	761	21.7
Washington	1,792	21.7
Madison	1,504	21.7
Scotland	476	22.1
Killingly	4,036	22.6
Cornwall	1,191	23.2
Voluntown	900	23.2
Farmington	4,272	23.7
East Granby	678	24.1
West Hartford	3,000	24.3
Wallingford	8,639	24.5
Glastonbury	4,140	24.8
Total population	82,208	
TOWNS AND CITIES WITH RATE 25–30		
East Lyme	1,868	25.1
New London	16,980	25.1
Windham	10,132	25.3
Hamden	4,514	25.6
Southington	5,832	25.8
Lisbon	675	25.9
Huntington	5,334	26.3
Westport	3,971	26.4
Stonington	8,326	26.4
Naugatuck	9,892	26.5
New Hartford	3,385	26.7
Redding	1,443	26.8
Ansonia	12,331	26.8

Community	Average Population 1890–1907	Average Typhoid Fever Rate 1890–1907
Kent	1,245	27.0
Thomaston	3,282	27.0
Plymouth	2,726	27.1
Brookfield	1,037	27.4
Waterford	2,867	27.7
Orange	6,626	27.8
Andover	387	28.1
Middletown	17,143	28.2
New Britain	26,823	28.6
Windsor	3,515	29.0
Norwalk	19,563	29.2
Putnam	7,224	29.3
Manchester	10,245	29.3
Bethlehem	568	29.5
Total population	187,949	
TOWNS AND CITIES WITH RATE 30–40		
Oxford	944	30.1
Old Saybrook	1,442	30.3
Bozrah	830	31.1
Bloomfield	1,482	31.4
Clinton	1,421	31.5
Danbury	19,474	31.7
Enfield	6,774	31.9
Somers	1,565	32.1
Haddam	2,027	32.5
Hebron	1,020	32.6
Branford	5,518	33.0
Woodstock	2,127	33.6
Brooklyn	2,400	34.0
North Canaan	1,786	34.9
Simsbury	2,060	35.3
New Haven	104,730	35.4
Stamford	18,365	36.1
Ridgefield	2,567	36.3
Bethel	3,338	39.5
Total population	179,870	
TOWNS AND CITIES WITH RATE 40–50		
Sterling	1,086	40.6
Hartford	75,857	40.8
Stafford	4,333	41.7
Sherman	658	41.9
Waterbury	48,448	42.1
Bristol	9,304	43.1
Eastford	529	43.4
Hampton	630	44.1
Killingworth	641	44.5
East Hartford	6,113	45.5
Ledyard	1,228	46.1
Watertown	2,983	46.1
Total population	151,810	
TOWNS WITH RATE OVER 50		
Durham	880	50.8
Cromwell	2,024	52.6
Roxbury	1,064	55.3
New Fairfield	596	72.6
Total population	4,564	

Here again, as in Massachusetts, we find some of the smallest communities among those having the least typhoid fever mortality. If the word "rural" has any meaning, it is certainly applicable to many of these small communities in which the typhoid fever rate has over many years been exceedingly low. It is true that an equal number of communities of similarly small population may be found among those groups having higher death-rates. But while it is easy to account for this fact by the occasional importation of one or more cases, it is not easy, it is rather impossible, to account for the prolonged absence of the disease from purely rural communities if typhoid fever is in fact a rural disease, i.e., a disease of rural origin and rural habitat.

The quantitative relation of population to typhoid fever in Connecticut is well brought out in the following table from which it appears (in confirmation of the Massachusetts results given on p. 174) that the lowest rates occur in the smallest communities. So likewise do the highest rates; but while we have between rate 0 and rate 10 a population of 32,508 in communities under 5,000 population, we have at rate 50 and higher only 4,564 souls in communities of 3,000 or less. Again, while it is true that the largest groups of the more rural population (under 1,000) is between rate 10 and rate 15, the largest urban group is at rate 30–40. These figures surely do not indicate that typhoid fever is a rural disease.

CONNECTICUT COMMUNITIES ARRANGED BY POPULATION GROUPS AND ANNUAL TYPHOID FEVER DEATH-RATES FOR THE YEARS 1890–1907, INCLUSIVE.

Typhoid Death-Rates at or between	Average Population of Communities, 1890–1907						
	Under 1,000	1,000–2,000	2,000–3,000	3,000–5,000	5,000–10,000	10,000–25,000	Over 25,000
0	2,663	0	0	0	0	0	0
0–5	0	0	2,764	3,450	0	0	0
5–10	1,719	7,033	7,160	7,719	0	0	0
10–15	7,713	11,923	9,766	19,742	0	0	67,676
15–20	5,357	12,394	9,872	20,066	14,429	35,932	28,176
20–25	4,975	10,727	5,146	19,484	30,111	11,865	0
25–30	1,630	5,593	5,593	18,682	43,234	86,394	26,823
30–40	1,774	8,176	11,181	3,388	12,292	37,839	104,730
40–50	2,458	2,314	2,983	4,332	15,417	0	124,305
Over 50	1,476	1,064	2,024	0	0	0	0
Totals	29,465	59,664	56,489	96,806	115,483	172,030	351,710

It will be remembered that the Massachusetts results indicated that previous to 1890 typhoid fever was far more truly than since that time "a rural disease" (p. 165). The following table reveals

the same fact for Connecticut communities and proves beyond peradventure that an astonishing change in urban and rural typhoid has occurred since about 1882. This table should first be studied by itself and then its last column (of 8-year averages) should be compared with the data for 1890–1907 on the table previously given:

TYPHOID FEVER DEATH-RATES (PER 100,000 OF POPULATION) IN CONNECTICUT COMMUNITIES GROUPED ACCORDING TO POPULATION. COMPUTED FROM THE CONNECTICUT STATE REGISTRATION REPORTS, 1882–1889, INCLUSIVE.

Population Group	Average Population 1882–1889	Rates by Years								
		1882	1883	1884	1885	1886	1887	1888	1889	Average Rate, 8 Years
Under 500 persons...	2,549	44.6	0	79.5	36.0	350.7	0	44.3	93.0	81.0
500 to 1,000.........	25,390	71.7	52.5	45.9	16.9	46.3	35.0	41.6	44.2	44.3
1,000 to 1,500.......	37,774	58.5	50.1	20.4	46.3	26.2	27.4	43.7	34.9	38.4
1,500 to 2,000.......	35,516	35.5	40.0	44.7	56.5	61.4	20.5	37.4	49.6	43.2
2,000 to 3,000.......	61,938	50.0	37.1	28.3	22.6	25.5	23.7	43.3	35.3	33.2
3,000 to 4,000.......	62,605	43.4	41.1	28.6	31.4	37.1	17.9	51.1	47.3	37.2
4,000 to 5,000.......	23,355	187.8	59.0	73.9	22.2	35.1	42.4	56.7	37.1	64.3
5,000 to 8,000.......	78,656	56.3	39.1	41.1	33.1	40.1	32.0	50.9	40.9	41.9
8,000 to 10,000.......	23,533	29.5	92.2	40.5	66.1	30.1	36.8	43.2	27.5	45.7
10,000 to 15,000......	57,031	35.1	28.1	25.6	14.7	14.6	23.1	18.9	21.2	22.4
15,000 to 25,000......	95,630	53.0	72.3	37.7	32.7	31.9	25.9	23.5	46.7	40.5
25,000 to Bridgeport..	61,413	27.2	5.7	49.7	38.1	27.5	34.7	27.9	24.2	29.4
Hartford and New Haven............	124,696	47.9	38.5	58.2	30.8	36.4	24.7	48.2	37.0	40.2
The state.........	690,086	49.6	44.0	40.7	32.5	34.6	27.1	40.4	38.3	38.4

Population calculated from U.S. Census figures.

Grouping the Connecticut communities according to their typhoid death-rates for the period 1882–89, brings out clearly the excessive incidence of typhoid fever in the towns below 5,000 population. A comparison of this table with the corresponding one for 1890–1907 shows a relatively greater decrease in typhoid fever mortality during the later period of our study for towns below 5,000 population than for the more urban communities above 5,000. In the group comprising the largest cities (over 25,000) a majority of the population was above "rate 30" in both periods. In the next two groups (5,000–10,000 and 10,000–25,000) a majority of the population was above "rate 30" in the earlier period and above "rate 25" in the later. More than half of the population of the groups below 5,000 was above "rate 30" for 1882–89 but the dividing rate had dropped to "rate 20" in 1890–1907. It appears then that in the 25 years under consideration the typhoid fever mortality

of the large cities of Connecticut has remained nearly constant, but that since about 1890 there has been a slight decrease in the typhoid rates of the communities of middle size and a considerable decrease in typhoid rates in the small towns.

CONNECTICUT COMMUNITIES ARRANGED BY POPULATION GROUPS AND TYPHOID FEVER DEATH-RATES (PER 100,000) FOR THE YEARS 1882–1889, INCLUSIVE.

Typhoid Death-Rates at or between	Average Population of Communities, 1882–1889						
	Under 1,000	1,000–2,000	2,000–3,000	3,000–5,000	5,000–10,000	10,000–25,000	Over 25,000
0	4,256	2,817	4,586	0	0	0	0
0–10	0	1,394	0	0	0	0	0
10–15	0	4,967	9,927	3,352	0	13,642	0
15–20	3,784	4,383	4,767	3,998	13,144	28,203	0
20–25	2,651	2,873	7,554	11,169	23,416	0	39,883
25–30	1,884	2,324	13,131	10,474	9,124	13,719	0
30–40	942	25,012	7,426	18,079	7,000	58,540	76,521
40–50	4,264	11,847	2,225	10,642	5,729	32,202	47,925
50–75	4,321	9,932	9,725	26,004	29,863	0	27,383
75–100	2,990	4,540	0	4,546	5,343	0	0
Over 100	2,319	1,538	2,410	0	6,978	0	0
Totals	27,417	71,627	61,751	88,265	100,597	146,306	191,712

6. EVIDENCE DERIVED FROM THE VITAL STATISTICS OF THE STATE OF NEW HAMPSHIRE.

Lastly we have carefully considered the vital statistics of the state of New Hampshire, comprising a number of communities closely similar in many respects to those of the contiguous state of Massachusetts and therefore not very different from those of the nearby state of Connecticut, but having a much larger proportion of its population scattered over a large area in smaller communities. There is also considerable difference in the economic and industrial conditions of New Hampshire communities, since manufacturing, which is so prominent in Massachusetts and Connecticut, plays a distinctly less important part in New Hampshire. All this means that New Hampshire is, on the whole, a more rural state than either of the others and that it has more of its population living under strictly rural, i.e., country, conditions. The population of New Hampshire, smaller than that of Massachusetts, is distributed over a slightly greater area, and is, accordingly, a sparser and less concentrated population.

In the following table we present certain vital statistics of New Hampshire arranged in a form similar to that employed above for the states of Massachusetts and Connecticut, and fortunately also, like these, based upon trustworthy registration reports:

YPHOID FEVER DEATH-RATES (PER 100,000 OF POPULATION) IN NEW HAMPSHIRE COMMUNITIES GROUPED ACCORDING TO POPULATION. COMPUTED FROM THE NEW HAMPSHIRE STATE REGISTRATION REPORTS, 1890–1907, INCLUSIVE.

Population Group	Number of Communities	Average Population 1890–1907	Rates by Years																		
			1890*	1891	1892	1893	1894	1895	1896	1897	1898	1899	1900*	1901	1902	1903	1904	1905	1906	1907	Average Rate, 18 Years
Less than 500 persons	51	15,520	35	18	36	18	30	68	31	6	13	13	20	27	..	7	35	7	14	14	21.8
500 to 1,000	81	57,810	27	49	27	22	39	15	29	10	16	21	24	16	14	18	11	5	16	5	20.2
1,000 to 1,500	43	51,790	37	17	17	17	17	10	27	10	21	23	19	15	13	19	21	13	12	8	17.6
1,500 to 2,000	24	41,130	40	58	40	22	37	20	25	20	19	19	36	34	17	12	19	21	12	21	26.2
2,000 to 3,000	13	31,430	25	25	13	16	38	10	13	13	25	10	3	16	22	29	..	6	25	9	16.5
3,000 to 4,000	7	23,760	58	57	51	64	18	4	31	22	38	54	41	12	20	55	16	4	30	19	33.0
4,000 to 5,000	2	9,610	12	36	36	..	68	22	33	54	11	10	40	..	20	10	..	19	9	9	21.6
5,000 to 8,000	3	18,840	63	25	48	59	17	11	39	11	27	16	5	20	30	15	43	14	14	18	26.4
8,000 to 10,000	3	26,080	33	28	12	36	36	20	35	27	15	4	11	19	19	15	15	18	11	11	20.3
10,000 to 15,000	2	23,660	18	22	31	26	17	17	13	13	25	17	25	29	29	25	12	25	33	28	22.5
15,000 to 25,000	2	37,450	30	54	24	18	23	33	32	12	21	9	5	11	13	13	15	11	18	3	19.2
Manchester	1	55,060	34	42	11	29	47	41	39	23	26	20	16	15	13	15	21	17	9	9	23.7
The state	232†	397,110	33.9	37.9	25.7	26.0	31.8	21.7	28.7	16.0	21.7	18.5	19.4	18.1	16.7	19.0	16.9	13.2	15.7	11.4	21.8

* Census years. Populations for intervening years estimated by method of U.S. Census.

† Two towns are omitted on account of changes in incorporation; also certain unincorporated places; total population thus omitted, 4,970.

From the above data we have prepared Fig. 5, in which the same method of presentation is used as for the other states, but in which both vertical and horizontal scales differ from those employed for Massachusetts and Connecticut.

Fig. 5 shows at a glance that the distribution of typhoid fever mortality in this state is distinctly different from its distribution in Massachusetts and Connecticut. The death-rates for the various

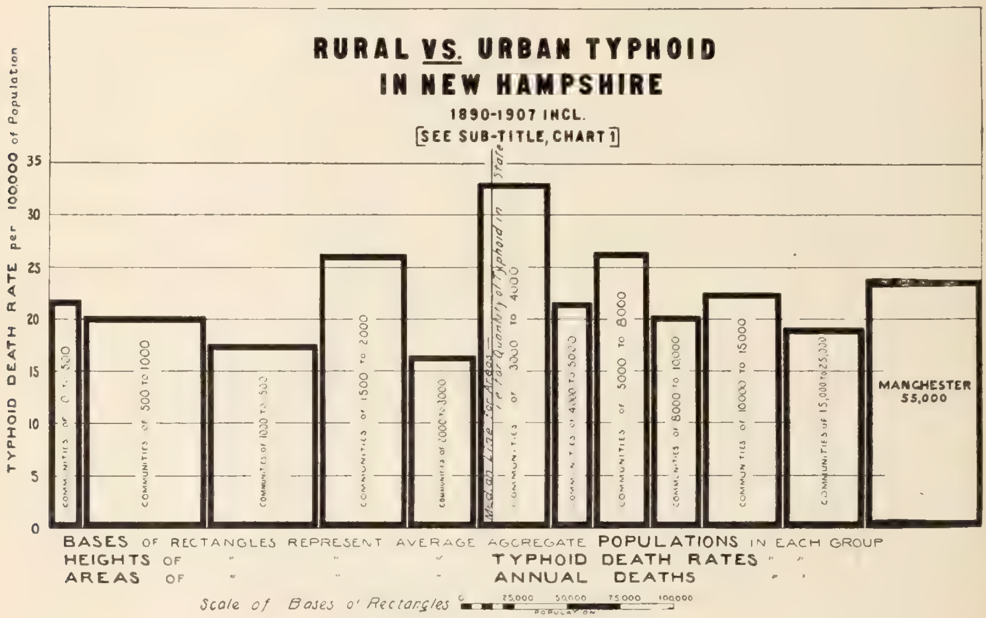


FIG. 5.

groups show marked irregularity, the minimum, for example (16.5 for communities of 2,000 to 3,000) standing directly next to the maximum (33 for communities of 3,000 to 4,000). In general, however, the death-rates at both ends of the population scale are low, while the rates are relatively high in the communities of medium size, notably those of 1,500–2,000, 3,000–4,000, and 5,000–8,000.

Considering the areas of the rectangles, that is, the total deaths from typhoid fever, the distribution appears to be fairly regular, the position of the median line indicating that about as many deaths occurred in places of more, as in those of less, than

3,000 population. This finding is quite different from that in Massachusetts and Connecticut, as will readily appear by comparing the median lines for those states with that for New Hampshire. At the same time the greater proportion of population in the smaller and more rural communities in the case of New Hampshire must not be overlooked. In New Hampshire, apparently, typhoid fever cannot be said to prevail to any decidedly greater extent in either group, rural or urban.

The fact that it appears to have been relatively abundant in communities of medium size, as noted above, is precisely what theory would lead us to expect in a state of this kind. We should not expect to find typhoid fever abundant in strictly rural districts of very small population and consisting chiefly of scattered farmhouses. Neither, on the other hand, should we expect to find it abundant in cities like Manchester, Concord, and Nashua, which are well known to have water-supplies of excellent quality and boards of health of good repute. We should rather look for it exactly where we find it most prevalent, namely in places which are neither strictly rural nor strictly urban, where people are perhaps gathered closely together, but as yet without the advantages of strictly urban conditions. In such places, as we have repeatedly intimated, modern conveniences, such as public water-supplies and sewerage systems, have often not yet come, although they are greatly needed, while the conditions are those of aggregation rather than segregation and carriers as well as unfavorable sanitary conditions naturally make for the prevalence of typhoid fever.

7. RECAPITULATION AND CONCLUSIONS.

In the first section of the present paper we have drawn attention to the discrepancy which appears to us to exist between the logical corollaries of our modern knowledge of the origin and distribution of typhoid fever on the one hand, and prevalent opinion concerning the relative frequency of typhoid fever under urban and rural conditions on the other.

In the second section we have cited at considerable length various authors, some of whom assert that typhoid fever is a rural

disease and others of whom appear to be equally confident that it is an urban rather than a rural disease.

In the third section we have discussed at some length the terms "urban" and "rural" in order to obtain a sound basis for such investigations as appear to us to be required.

In the fourth section we have described in detail a very careful and, as we believe, a thorough statistical investigation of the actual facts concerning the distribution of typhoid fever mortality in country and city in the state of Massachusetts; and in the two following sections, similar studies of the vital statistics of the contiguous states of Connecticut and New Hampshire.

It now only remains to bring together what we conceive to be the results of all these studies and to draw from them such deductions as are justified.

The first and most important conclusion to which our investigations lead is, that if we take as long a period as 18 years previous to 1908, typhoid fever cannot be said to be in any real and true sense of the word distinctly a rural disease, that is to say, preeminently a disease of small communities composed of isolated or scattered dwellings distributed over country districts.

A second and perhaps equally important result is that this general finding, while it holds good for the period 1890–1907, is not true if we consider only the preceding decades. In other words, about 1890 a remarkable change appears to have taken place in the incidence of typhoid fever mortality—at least in Massachusetts and in Connecticut—such that whereas previous to that time typhoid fever deaths were more prevalent in proportion to the population in the country than in the city districts, after that time and measured in that way typhoid fever became distinctly a more urban than rural disease. For this discovery we were in no wise prepared when we began our studies and we are the more gratified to have developed the fact because it enables us to harmonize what would otherwise have been our discordant results with the studies and statements of earlier authors.

It is not easy to account for this change, and we might perhaps better leave our results as they stand, without trying to explain them. Piqued by curiosity, we have, however, sought to find some

explanation of them, first, for the fact—for it appears to be a fact—that previous to 1890 typhoid fever in Massachusetts and Connecticut evidently was a rural rather than an urban disease, second, for the change which then took place, and, third, for the present state of affairs in which typhoid fever (in Massachusetts and Connecticut at least) appears to be an urban rather than a rural disease.

It is hard today to see why typhoid fever should ever be, or ever have been, more abundant in country than in city. The very fact of segregation of population tends to make infection difficult, and without infection there can be no typhoid fever. It was this *a priori* reasoning which originally led us to undertake the present investigation, for this view is opposed *in toto* to the popular belief that typhoid fever is a rural disease, and, as we have stated at the beginning of this paper, it was this contradiction between rational theory and traditional opinion which caused us to set to work to discover the truth.

We know that before 1890 typhoid fever in Massachusetts and Connecticut was apparently more a rural than an urban disease, not only from the studies reported in the present paper but also from the investigations of Dr. Abbott and Dr. Derby. The question arises (1) Why was this the case? And also, (2) What conditions of *infection*, *virulence of microbes*, or *vital resistance* (the three factors of any infectious disease) prevailed before the year 1890 or thereabouts which were afterward so altered as to reverse the incidence of the disease?

There is no good reason to assume for the years previous to 1890, any greater virulence of typhoid microbes in the country than in the city, or any greater vital resistance in the city than in the country, nor is there any reason for assuming any change in relative virulence or relative resistance since that time. The only possibility that has even a shadow of reasonableness is that somehow the relative conditions of infection have changed. But if so, how? There is one possible explanation which, though not wholly satisfactory, is worthy of note:

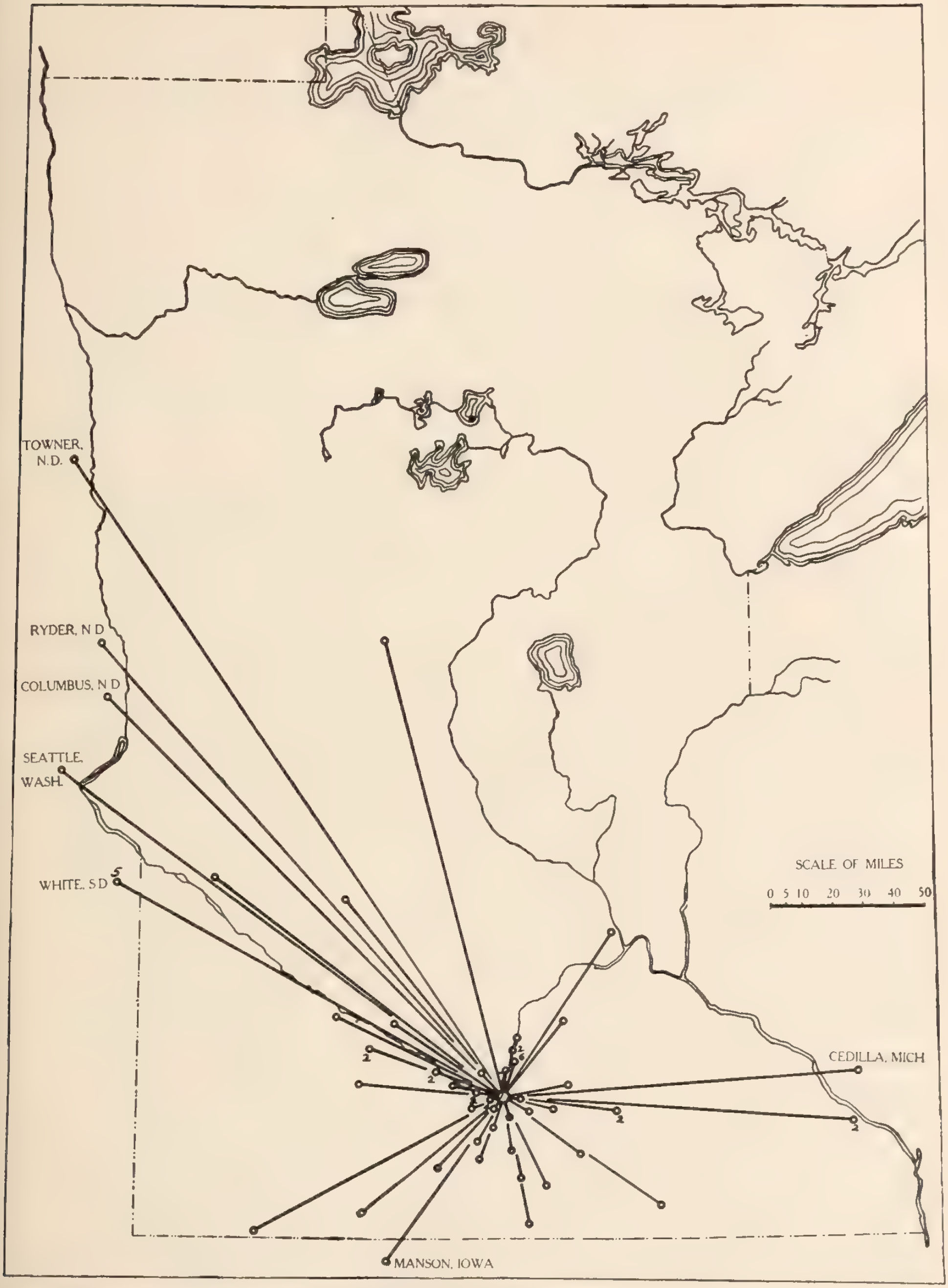
The Civil War of 1861–65 caused the germs of typhoid fever to be carried as never before or since into many a rural home

and many a rural hamlet. Some, perhaps many, of those who came back from the war worn and weakened were doubtless "typhoid carriers," and some doubtless long remained such. It is possible that the infection thus widely planted continued to be felt during the 30 years between 1861 and 1891, but by the latter date had become mostly attenuated or extinct. If this were true we should only have to explain the relatively higher mortality in the rural regions during the period. And this might be done by supposing (what was doubtless true) that various rural conditions, such as more frequent failure to recognize or attend to the disease, less skilful diagnosis, or nursing, or medical attendance, or more contacts *within families*, etc., existed in the more rural districts.

Assuming the truth of this hypothesis of lingering military or camp infection from the Civil War, it is comparatively easy to account for all the facts. The presence of high typhoid rates in the country districts during the periods studied by Drs. Derby and Abbott—a condition normally impossible—would be due to the return of infected soldiers. The same cause should also produce at the same time high typhoid rates in cities, but some difference in favor of a lower rate in the cities might have been due to early diagnosis, better care, hospital treatment, etc. The gradual decline in both urban and rural typhoid rates might be due in part to the gradual exhaustion of the original infection. A more rapid decline in the country than in the city might be expected to occur because of the comparative isolation of the patients and the absence of opportunities for contact infection outside infected families or houses.

On this theory also we have an explanation in whole or in part of the origin of the high typhoid fever death-rate which has long been a reproach to the entire United States. The prolonged persistence of that high rate, however, could hardly be due wholly to such original infection. Other factors, such as polluted water, polluted milk, promiscuity, etc., must be called in to explain such persistence, and these would be much more likely to be effective under urban than under rural conditions.

The effect of certain other great movements of the population during the period covered by our study deserves careful consideration, namely, *immigration* and *urbanization*, or the growth of cities more or less at the expense of the country.



Map of Minnesota showing the position of Mankato and how typhoid cases were radiated from it during the epidemic.

It is probably true that during the period under consideration immigration of Europeans free from typhoid fever was directed largely to the more urban portions of New England, and this was probably especially true during the earlier period, which would only tend to bring out more clearly the phenomenon of typhoid fever excess in the rural districts. In the later years of the period more of this typhoid-free population has perhaps gone to the country and may thus have helped to accentuate that decline in rural typhoid which we have repeatedly noted. Obviously, the general effect of such immigration would be to dilute the population with typhoid-free material and thus help to lower the general rate. It is, however, quite impossible to be certain how far the inflow of immigrants from Europe has influenced, if at all, the American death-rate from typhoid fever.

The other great movement, from the country districts into the cities, has been equally marked and very likely equally influential. But here also it is impossible to say precisely what the effect of this movement upon typhoid fever incidence and mortality may have been.

It will doubtless appear strange and incredible to many not only that typhoid fever is today an urban rather than a rural disease, but also that it is quite as often spread from town to country as from rural to urban districts. We are therefore glad to be able to refer for an illustration and a striking example to the epidemic of 1908 at Mankato, Minn., first described by Professor F. H. Bass in the *Engineering News* of February 11, 1909 (61, p. 151).

In the Mankato epidemic, which was due to polluted water, 72 cases with five deaths were found in the surrounding country which had derived their infection from the polluted water-supply of Mankato. A diagram of some of these was originally published by Professor Bass (*op. cit.*) in which the sites of the cases are connected with Mankato by lines radiating from the city as a center to the various rural points, some near and some very remote, where the cases occurred. The diagram is so striking and so instructive that we have ventured to reproduce it here. A complete account of the epidemic and another map appeared in this *Journal* for 1911, 9, p. 422, Fig. 6.

SPOROTRICHOSIS IN THE UNITED STATES.*

GUSTAV F. RUEDIGER.

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Ten years ago we were of the opinion that infection of the human subject with the sporothrix fungus was so rare as to be a pathological curiosity. At that time only three cases had been mentioned in the literature of America and no cases had been recognized in any other country. During the last six years, however, more than 50 cases have been reported from France, one or two cases have been observed in Germany, and 44 additional cases have been reported from different parts of the United States. In addition to these, I have been able to collect 10 new cases from the United States which have not yet been reported (some of these will be reported by those in whose practice they occurred), making a total of 57 cases thus far observed in the United States.

My attention was called to the existence of this disease in North Dakota by the report of a case from Braddock, N.D., by Hyde and Davis[1] about two years ago. The description of this case recalled to my mind a conversation with Dr. E. P. Quain of Bismarck, who had been treating similar cases and had reported six of these as cases of "Tubercular Ulcers and Tubercular Lymphangitis of the Upper Extremity." Dr. Quain was not satisfied with that diagnosis and we have shown since that it was incorrect, and that these were, in fact, cases of sporotrichosis.[2] During the last two years, I have been engaged in making a careful study of the prevalence of this disease in North Dakota and have been able to collect 22 authentic cases, six of which were diagnosed culturally.

While getting together the histories of the cases in North Dakota, I was impressed by the fact that all the cases that have been observed were found in a definitely limited strip of territory along the Missouri River. This fact led me to make a careful study

* Received for publication July 29, 1912.

[1] American Dermatological Association, Washington, May, 1910.

[2] Ruediger and Miller, *Northwestern Lancet*, 1911, 31, p. 507.

of the geographical distribution of all the cases reported from the United States and I found that approximately five-sixths of the observed cases are from the Missouri Valley. This fact will be seen at a glance by referring to the accompanying map where the location of each case is indicated by a dot.

Only 24 of the 57 cases I have collected from the United States were confirmed by a cultural or microscopic diagnosis. It is possible that some errors in diagnosis have crept in, although the histories and descriptions do not indicate that this is the case. In view of this fact, however, the following very brief abstracts of all the case histories may serve a useful purpose in this connection. They are grouped according to the states in which they occurred.

MISSOURI.

Case 1.—The first case of sporotrichosis that has been recognized is that of Schenck.[1] The patient, male, aged 36, was working in an iron foundry in St. Louis at the time infection occurred. The infection started from a scratch on the finger, made by an old nail. Three weeks after the injury was received, an ulcer appeared higher up on the finger and in seven weeks seven similar abscesses formed in the lymph channels on the radial side of the arm. Cultural diagnosis was made.

Case 2.[2]—The patient, a farmer's daughter, aged 18, was a resident of Independence, Mo. Infection started from a slight wound on the back of the right hand. Several weeks later two small subcutaneous abscesses developed, one on the wrist and one higher up on the forearm. Cultural diagnosis was made.

Case 3.[3]—The patient, a farmer's daughter, aged 16, lived at Highpoint, Mo. Infection started in an incised wound on the index finger on the right hand made by a kraut-cutter. Later a chain of 11 nodules developed on the ulnar side of the forearm and on the inner side of the upper arm. Two of those on the forearm broke down and ulcerated. Patient recovered under potassium iodide treatment. No cultural diagnosis was made.

KANSAS.

Case 4.[4]—The patient, aged 69, was a laborer by occupation and a resident of Kansas City. The infection started as a small pimple over the point of insertion of the deltoid muscle. This developed into a small ulcer, and others soon made their appearance until there were 15 ulcers and eight cutaneous nodules over the region of the deltoid muscle. Cultural diagnosis was made.

Case 5.[5]—The patient, female, aged 30, was a resident of Ottawa, Kan. She experienced a pricking sensation in the thumb and a few days later a reddened papule

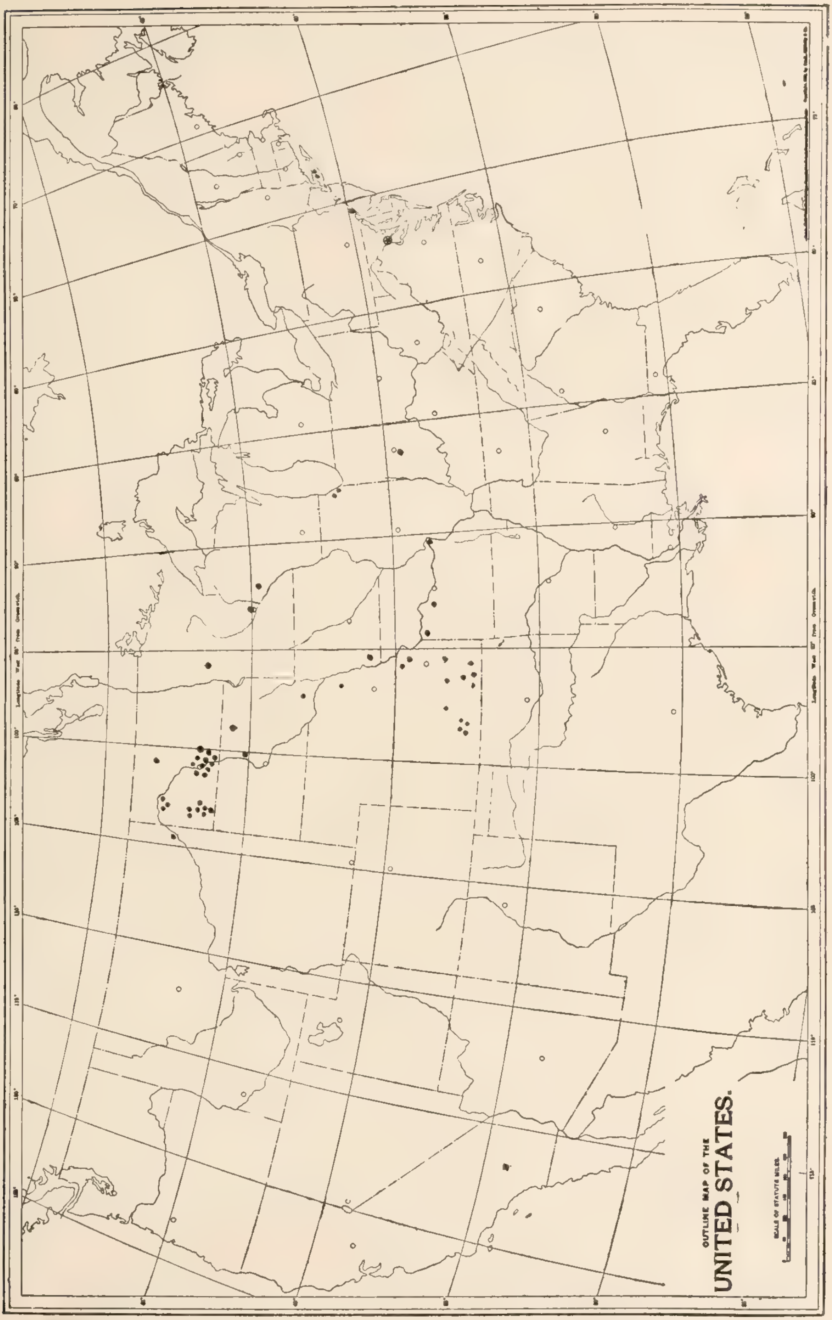
[1] *Johns Hopkins Hospital Bulletin*, 1898, 9, p. 286.

[2] Richard L. Sutton, *Jour. Am. Med. Assoc.*, 1910, 55, p. 2213.

[3] Richard L. Sutton, *ibid.*

[4] Trimble and Shaw, *Jour. Kansas Med. Society*, 1909, 9, p. 305.

[5] Richard L. Sutton, *Jour. Am. Med. Assoc.*, 1910, 55, p. 1000.


OUTLINE MAP OF THE
UNITED STATES.

developed on this area. This gradually increased and finally developed into an ulcer which was sharply outlined and free from pain. Four weeks later, a hard cutaneous nodule developed near the base of the thumb. At intervals of a few days, six other nodules developed higher up on the arm, extending from the thumb to the shoulder. Culture was isolated.

Case 6.[1]—The patient, a farmer's boy, aged 11, was a resident of Tyro, Kan. Infection started in a hen bite on the back of the hand. About four weeks after the injury was received, abscesses began to form on the forearm, and in the course of two months, 21 of these abscesses developed. No microscopic examination or cultural diagnosis was made.

Case 7.[2]—The patient, aged 45, was a farmer by occupation and a resident of Earlton, Kan. He had on his premises a horse that was probably afflicted with sporotrichosis. While repairing a manger in the barn, he sustained a punctured wound on the wrist from a piece of baling wire. The wound did not heal but increased in size and later small subcutaneous nodules began to appear on the flexor side of the forearm and upper arm. Ten of these nodules appeared within six weeks. Cultural diagnosis was made.

J. M. Sutton,[3] of Halstead, Kan., has reported the following four cases:

Case 8.[4]—The patient, aged 52, was a laborer by occupation. He punctured the palm of his left hand with a nail while tearing down an old barn. The wound healed without trouble but three weeks later a nodule developed on the dorsum of the hand opposite the site of the puncture. Within a week after this nodule had developed, 24 similar small nodules appeared higher up on the arm. A microscopic examination of the pus showed the presence of *Sporothrix schenkii.*

Case 9.[5]—The patient, aged 40, a farmer by occupation. He cut the index finger on the right hand with a disc harrow. The injury refused to heal and two weeks later showed signs of infection. Within two months, more than 50 small characteristic subcutaneous nodules appeared on the hand, forearm, and upper arm. No cultural or microscopic diagnosis was made.

Case 10.[6]—The patient, aged 35, was a farmer by occupation. He received a slight cut on the thumb which healed readily. Two weeks later the scar became inflamed and nodular enlargements developed on the dorsum of the hand. No cultural or microscopic diagnosis was made.

Case 11.[7]—The patient, aged 28, was a farmer by occupation. Infection started in a small pimple on the arm. This broke down and ulcerated and later seven small tumors appeared on the right upper arm. No cultural diagnosis was made.

All four of these cases were treated as sporotrichosis and made a rapid recovery under internal medication with potassium iodide.

[1] Richard L. Sutton, *Jour. Am. Med. Assoc.*, 1910, 55, p. 2213.

[2] Richard L. Sutton, *Boston Med. and Surg. Jour.*, 1911, 164, p. 179.

[3] *Jour. Am. Med. Assoc.*, 1911, 56, p. 1309.

[4] *Ibid.*

[5] *Ibid.*

[6] *Ibid.*

[7] *Ibid.*

Case 12.[1]—The patient, aged five, was a farmer's son, residing near Horton, Kan. Infection started in a scratch on the back of the hand which began to ulcerate. Later small nodules developed on the forearm and eventually broke down and formed ulcers. No cultural or microscopic diagnosis was made.

Case 13.[2]—The patient, aged17, was a farmer by occupation, residing near Wiley, Kan. Infection started in the form of a small papule on the left knee. Four nodules developed within a month higher up on the inner side of the thigh. No cultural or microscopic diagnosis was made.

Case 14.[3]—The patient, a woman aged 42, was a resident of Dennison, Kan. The infection started in the form of a papule on the middle of the right forearm. Within a short time afterward, seven small subcutaneous nodules appeared on the arm, two of which broke down and ulcerated. No cultural or microscopic diagnosis was made. Patient made a rapid recovery under potassium iodide treatment.

Case 15.[4]—The patient, aged 19, was a farmer by occupation, residing near Erie, Kan. The infection started in a small pimple on the dorsum of the left hand, gradually enlarged, and developed into an ulcer. The lymphatics leading up the arm became swollen and from 18 to 20 subcutaneous nodules developed in their course. A microscopic examination of the pus showed the presence of *Sporothrix schenkii.*

IOWA.

Case 16.[5]—The patient was a boy aged five, a resident of Shenandoah, Ia. The infection started in an abrasion on the finger which was sustained by a blow with a hammer. The abrasion began to ulcerate and 20 subcutaneous nodules developed in the lymphatics extending up the arm. A number of these broke down and formed ulcers. Cultural diagnosis was made.

NEBRASKA.

H. Gifford,[6] of Omaha, Neb., has reported the following six cases, only one of which, however, was diagnosed culturally.

Case 17.[7]—Infection in this case occurred on the eyeball and conjunctival surface of the eyelids of a young woman. Cultural diagnosis was made.

Case 18.[8]—The patient was a girl aged 14. Parents had noticed a growth in the right lower eyelid during the past month. At first there was only a small pimple but when seen by the physician there was found on the outer three-fifths of the lower lid a rough mass of granulations, elevated one-eighth of an inch above the surface, one-fourth of an inch broad at its outer extremity, and one-eighth of an inch broad at its inner extremity. Granulations were scraped out with a spoon and the wound cauterized. Recovery. No cultural diagnosis was made.

[1] Harry J. Harker, *Jour. Am. Med. Assoc.*, 56, p. 1312.

[2] Albert Beam, *ibid.*, p. 1790.

[3] W. B. Stewart, *ibid.*, 57, p. 482.

[4] Ralph C. Henderson, *ibid.*, 56, p. 1048.

[5] Hektoen and Perkins, *Jour. Exp. Med.*, 1900, 5, p. 77.

[6] *Oph. Record*, 1910, 19, p. 580.

[7] *Ibid.*

[8] *Ibid.*

Case 19.[1]—The patient was a boy aged 10. The infection started in the left lower eyelid which on examination presented two elevated areas of rough granulation tissue covered with slight crusts. One of these areas was just below the lashes and was one-fourth of an inch long and the other one, about twice as long as the first, was situated just below it. The granulation tissues were scraped out with a sharp spoon and iodiform dressing applied. Recovery.

Case 20.[2]—The patient was a woman aged 32. She had noticed a swelling below the left eye for about four months before consulting a physician. At the time of the examination the inner half of the left lower lid presented a mass of granulation tissue one-fourth of an inch in diameter, raised about one-eighth of an inch and covered with a thin crust. The granulation tissue was scraped out with a sharp spoon, an iodiform dressing applied, and the patient was given potassium iodide internally. Good recovery. No cultural diagnosis was made.

Case 21.[3]—The patient was a boy aged four and one-half. The infection started on the right lower lid in the form of a small reddish swelling. About the same time there appeared a painful lump at the angle of the right jaw. When seen by a physician, there was found on the inner side of the right lower lid an inch below the caruncle a reddish elevation five-eighths of an inch long and one-fourth of an inch broad. The epidermis was broken down in the center and the rest of the tumor was covered with smooth skin. A small amount of pus was squeezed out. The tumor was scraped out with a sharp spoon and the patient was given potassium iodide internally. Good recovery. No cultural diagnosis.

SOUTH DAKOTA.

Case 22.[4]—The patient was a girl aged four. When seen by the physician, there was a mass of granulation tissue on the lower border of the right tear sac extending two-thirds of the way to the outer angle of the lower lid. The tumor was thoroughly scraped out with a sharp spoon and did not reappear. No cultural diagnosis was made.

Case 23.[5]—The patient was a farm laborer by occupation and resident of Groton, S.D. The infection started in the form of a pimple on the inner side of the leg, several inches below the knee. Later five subcutaneous nodules developed higher up on the leg and lower portion of the thigh. Cultural diagnosis was made.

Case 24.[6]—The patient, aged 27, was a farmer by occupation, and a resident of Potter County, S.D. The infection started in a small fissure on the index finger which gradually enlarged and developed into an ulcer. Within the next two weeks, about 15 small subcutaneous nodules appeared on the dorsal side of the forearm and on the inner side of the arm, extending up to the axilla. Cultural diagnosis was made.

NORTH DAKOTA.

Case 25.[7]—The patient, aged 24, was a farmer by occupation, and a resident of Braddock, N.D. Infection started in the form of a papule on the back of the left hand.

[1] *Oph. Record*, 1910, 19, p. 580.
[2] *Ibid.*
[3] *Ibid.*
[4] *Ibid.*
[5] Walter Hamburger; reported to Am. Ass. of Path. and Bact., Chicago, April, 1911.
[6] G. S. Adams, Yankton, S.D. Case not yet reported.
[7] Hyde and Davis, *Jour. Cutan. Dis.*, 1910, 28, p. 321.

This began to ulcerate and later six nodules appeared on the left arm. Some time after the appearance of the nodules on the left arm, six similar nodules appeared on the lower third of the right arm, one on the right forearm, and one on the posterior surface of the leg near the knee. Cultural diagnosis was made.

Case 26.[1]—The patient, aged 45, was a farm laborer and a resident of Stark County, N.D. The infection started on the middle finger where he had received a prick from a straw. This developed into an ulcer and within a few weeks 21 subcutaneous nodules developed on the forearm and arm extending up to the axilla. Cultural diagnosis was made.

The following eight cases were treated by Dr. E. P. Quain of Bismarck and six of them were reported by him[2] as cases of "Tubercular Ulcers and Tubercular Lymphangitis of the Upper Extremity."

Case 27.[3]—The patient was a woman aged 35. The infection started in the form of a papule on the inner side of the leg. This developed into an ulcer and later a number of subcutaneous nodules appeared in the course of the swollen lymphatics leading up to the groin. Cultural diagnosis was made by Ruediger.

Case 28.[4]—The patient was a boy aged 5. The infection started in a bruise on the dorsum of the hand which became infected, and about a month later small nodules began to appear under the skin of the forearm. The nodules gradually softened and discharged pus and showed no tendency to heal. No cultural diagnosis was made.

Case 29.[5]—The patient was a girl aged 18, a country school-teacher by occupation. The infection started in a cut on the left thumb which began to ulcerate and refused to heal. About six weeks after the injury was sustained, a number of small tender nodules were discovered in the subcutaneous tissues of the forearm and arm. The majority of these nodules opened spontaneously and discharged a thin purulent material. No cultural diagnosis was made.

Case 30.[6]—The patient was a woman, aged 40. The infection started from a small abscess on the back of the left hand from which developed an acute cellulitis of the arm and a lymphangitis of the arm and forearm. The abscess was incised and the acute symptoms subsided but the ulcer refused to heal and a number of small pustules appeared on the posterior surface of the forearm. A month later a chain of small subcutaneous tumors appeared along the anterior surface of the arm near the elbow. No cultural diagnosis was made.

Case 31.[7]—The patient was a man aged 70. The infection started in the form of a small ulcer over the distal end of the radius. A month later, secondary nodules appeared in a line along the radial side of the forearm extending to the elbow. These nodules subsequently broke down and ulcerated. No cultural diagnosis was made.

Case 32.[8]—The patient was a woman aged 48. The infection started in a slight bruise on the posterior surface of the right forearm which developed into an abscess. The pus was evacuated but the ulcer refused to heal and within a month a number of small subcutaneous nodules appeared chainlike along the anterior surface of the forearm extending from the ulcer to the elbow. No cultural diagnosis was made.

[1] Ruediger and Miller, *Jour. Minn. State Med. Ass.*, 1911, 31, p. 507.

[2] *St. Paul Med. Jour.*, 1904, 6, p. 615.

[3] Ruediger and Miller, *loc. cit.*

[4] E. P. Quain, *loc. cit.*

[5] *Ibid.*

[6] *Ibid.*

[7] *Ibid.*

[8] *Ibid.*

Case 33.[1]—The patient was a girl aged three, a granddaughter of patient No. 32. The infection started from a small scratch on the wrist which the child had playfully bandaged with dressings that had been used on the ulcers on the grandmother's arm. No cultural diagnosis was made.

Case 34.[2]—No satisfactory history of this case is available. Infection involved the forearm and the arm and was characterized by the development of a number of small subcutaneous nodules which gradually broke down and ulcerated. Scrapings from the ulcers were sent to the Public Health Laboratory to be examined for tubercle bacilli with negative results.

Case 35.[3]—The patient was a girl, aged three and one-half, a resident of Dunn County, N.D. The infection started in a cut on the index finger of the left hand which became greatly inflamed and swollen. Several weeks later, about 10 subcutaneous nodules developed on the posterior surface of the forearm and arm. Cultural diagnosis was made.

Case 36.[4]—The patient was a woman aged 43, a resident of Dunn County, N.D. The infection started in the form of a pimple on the back of the hand which soon resulted in an extensive cellulitis of the hand and arm. The papule began to ulcerate and within several months 12 subcutaneous nodules developed along the course of the lymphatics extending to the axilla. Ten of these were incised and resulted in very refractive ulcers. No cultural diagnosis was made.

Cases 37, 38, and 39.[5]—Dr. V. H. Stickney refers to five cases that were seen by him in Dickinson, N.D. Two of these cases were treated by Dr. G. A. Perkins and three by Dr. Stickney, who sent me the following descriptions:

In one case which was observed in 1905, the characteristic nodules were confined to the hand and forearm and appeared in a row along the course of the lymphatics. In the second case which was treated by him in 1905, the lesions extended along the arm and upper arm to the axilla. The third case was seen by him in the spring of 1907. In this case, the nodules appeared on the right shoulder and the left side of the neck and face. No positive diagnosis was made in either case.

Case 40.[6]—The patient, aged 19, was a farm laborer by occupation and was living in Kidder County, N.D., at the time infection was contracted. The infection started in the form of a pimple on the back of the hand which began to ulcerate. The ulcer refused to heal and in the course of several weeks four nodules developed higher up on the forearm. Cultural diagnosis was made.

[1] *Ibid.*

[2] Not previously reported.

[3] Ruediger and Smith, *Journal-Lancet*, 1912, 32, p. 227.

[4] *Ibid.*

[5] *Jour. Minn. State Med. Ass.*, 1911, 31, p. 512.

[6] G. M. Olson. Not yet published. Will appear in the *Jour. Am. Med. Assoc.*

The following six cases were collected from North Dakota by the writer and have not been previously reported.

Case 41.[1]—The patient, a woman aged 20, was a resident of Morton County, N.D. The infection started in the form of a small pimple on the elbow, which gradually developed into a discharging ulcer. Five weeks after the initial sore was noticed, secondary nodules began to appear in rapid succession until five of these were found on different parts of the arm. Cultural diagnosis was made by Ruediger.

Case 42.—The patient, aged 32, was a farmer by occupation, living in McHenry County, N.D., and was in the care of Dr. J. T. Newlove of Minot. The infection started as a small swelling on the hand which gradually subsided but very soon afterward a red and inflamed area appeared between the thumb and index finger. About 10 days later, he consulted a physician who discovered a number of small subcutaneous nodules extending up the forearm, above the inflamed area. Cultural diagnosis was made by Mr. L. V. Parker of the State Public Health Laboratory.

Case 43.—This case was reported to me in a letter by Dr. John A. Johns of Hettinger, N.D. The patient was a woman, aged 42, residing in Adams County, N.D. The infection started in the form of a pimple on the inner surface of the forearm. Little attention was paid to this but others gradually developed until six of these nodules had appeared and began to break down and ulcerate. On examination, it was found that there was a chain of hard subcutaneous nodules extending along the upper arm to the axilla. No cultural diagnosis was made but the case was treated as one of sporotrichosis, and made a rapid recovery under potassium iodide.

The following three cases were reported to me in a letter by Dr. George C. Hanson of Charlson, N.D. In these cases, a clinical diagnosis of sporotrichosis had been made but no cultural diagnosis was made.

Case 44.—The patient was a boy aged four. The infection started in a slight scratch on the back of the hand which started to ulcerate and refused to heal. Three weeks later when seen by the physician, the ulcer was as large as a half-dollar, and a number of subcutaneous nodules were found under the skin of the forearm. The patient made a rapid recovery under potassium iodide treatment.

Case 45.—The patient was a man aged 48. The infection started as a pimple on the dorsum of the right hand and developed into a refractory ulcer. When seen by the physician, this ulcer was as large as a half-dollar and there was a chain of subcutaneous nodules along the radial side of the forearm and inner side of the arm extending to the axilla. A few of the nodules on the forearm had broken down and were discharging a thin purulent fluid. Patient made a rapid recovery under potassium iodide treatment.

Case 46.—Patient was a boy aged seven. The infection started on the back of the hand and developed into a small refractory ulcer. When seen by the physician about three weeks later, there was found a chain of 15 subcutaneous nodules extending along the dorsal surface of the forearm.

Case 47.[2]—The patient, aged 41, was a farmer by occupation and a resident of Poplar, Mont. The infection started in the tip of the index finger. The patient first

[1] Patient of Dr. H. Altnow, Mandan, N.D. Not previously reported.

[2] Patient of Dr. John A. Johnson, Bottineau, N.D. Not previously reported.

noticed a slight pricking sensation, and when the finger began to swell slightly, he thought he had a sliver of wood in it which he attempted to remove with a pin. A little pus escaped and as the finger was inflamed and swollen he poulticed it with fresh cow manure and continued this treatment for 12 days. After the finger had been poulticed for eight days, a cauliflower growth, the size of a hazelnut, developed at the site of the injury. About ten days later purplish nodules began to appear on the back of the arm, extending to the elbow. When seen by the physician, 15 of these nodules were present and one large abscess was discovered. Cultural diagnosis was made by Ruediger.

The remaining 10 cases occurred outside of the Missouri Valley and were widely scattered from the Atlantic to the Pacific coast as is shown by the abstracts below.

Case 48.[1]—The patient, aged 50, was a laborer by occupation and resident of St. Paul. His work necessitated the handling of green hides. The infection started in the form of a pimple on the back of the right hand, which gradually enlarged and finally developed into an ulcer. About three weeks later, another similar swelling appeared over the distal end of the ulna and later many others appeared higher up on the ulnar side of the forearm and arm. Cultural diagnosis was made.

Case 49.[2]—The patient was a woodsman by occupation and had been working in the vicinity of Frazee, Minn. The infection started as an ulcer on the back of the hand and in the course of several months more than 25 characteristic nodules and superficial ulcers developed on the forearm and arm extending chainlike up to the axilla. Cultural diagnosis was made.

Case 50.[3]—The patient was a young woman, resident of northern Wisconsin. The infection started in a small cut made with a knife, on the middle finger, while peeling potatoes. The finger became inflamed and a lymphangitis extended up the arm. The original injury began to ulcerate and for a period of one and a half years repeated nodules developed on the arm and forearm which gradually softened and began to ulcerate. No cultural diagnosis was made.

Case 51.[4]—The patient, aged 25, was an express packer by occupation and a resident of Chicago. The infection started in the form of a flat pimple or tumor on the leg which gradually enlarged and finally developed into a discharging ulcer. A number of these ulcers developed higher up on the leg. A cultural diagnosis was made.

Case 52.[5]—The patient was a woman aged 25, a florist by occupation, and a resident of Chicago. The infection started in the form of a cold abscess on the forearm which was followed by several similar abscesses higher up on the arm. No cultural diagnosis was made.

Case 53.[6]—In this case, which occurred in a male, the infection started in a punctured wound on the finger. The chain of secondary nodules and ulcers extended

[1] J. M. Armstrong, *St. Paul Med. Jour.*, 1912, 14, p. 218.

[2] Patient of Dr. J. A. Thabes, Brainerd, Minn. Not previously reported.

[3] Harry Ritchie, *Jour. Minn. State Med. Ass.*, 1911, 31, p. 511.

[4] K. A. Zurasky, *Jour. Cutan. Dis.*, 1910, 28, p. 350.

[5] W. A. Pusey, *Jour. Cutan. Dis.*, 1910, 28, p. 352.

[6] Brayton, Abstract by R. L. Sutton, *op. cit.*

from the finger to the elbow and finally healed with considerable scarring. No bacteriological diagnosis was made.

Case 54.[1]—The patient was a resident of Philadelphia. Infection started from an injury on the finger which was followed by a chain of subcutaneous nodules and abscesses along the lymphatics of the arm. No cultural diagnosis was made.

Case 55.[2]—The patient was a boy aged 12, an inmate in a children's home at Smithtown Branch, N.Y. When the infection was discovered, there was found an ulcer on the top of the great toe and another one on the instep of the same foot. There was also present at that time a bluish, sharply defined nodule on the back of the wrist. This nodule fluctuated, and when opened, discharged a thick tenacious brownish pus which, on microscopic examination, showed the presence of *Sporothrix schenkii.* During the next month, two other subcutaneous nodules developed on the forearm. Patient was put on potassium iodide treatment and rapidly improved but left the children's home before the ulcers had entirely healed and had a relapse. At this time, about 20 ulcers and sores developed on his body.

Case 56.[3]—This case occurred in the same children's home as did the preceding case. The infection started in the form of a purplish nodule on the back of the hand and was followed very soon by a similar nodule on the wrist. Microscopic diagnosis was made.

Case 57.[4]—The patient was a farm laborer by occupation and a resident of Santa Ana, Cal. The infection apparently started on the left leg but later involved the right leg and also the face. Cultural diagnosis was made.

I am well aware that this series of reported cases is rather small for drawing general conclusions in regard to the geographical distribution of this disease. It is a very significant fact, however, that five-sixths of the total of 57 cases have been observed in the Missouri Valley. Another very important point is our finding in North Dakota. I have been able to collect 22 authentic cases from this state and every one of these occurred along the Missouri River. Not a single case in North Dakota has thus far been found outside of the Missouri Valley. It appears, therefore, that this organism is more commonly found along the Missouri River than in any other part of the United States.

It is an interesting question whether the organism lives as a saprophyte upon grains, grasses, or other vegetation, from which the infection is contracted, or whether each case is related directly to another pre-existing case. It seems, however, that the former must be the case because the disease is found most commonly

[1] Stelwagon, *Jour. Cutan. Dis.*, 1910, 28, p. 252.

[2] Guy H. Turrell, *Long Island Med. Jour.*, 1911, 5, p. 484.

[3] *Ibid.*

[4] Burlew, *Southern California Practitioner*, 1909, 24, p. 1.

among farm laborers and the cases are nearly always isolated. I know of only two instances where a second case developed in an individual who had associated with a person suffering from this infection. One of these was Dr. Turrell's case in the children's

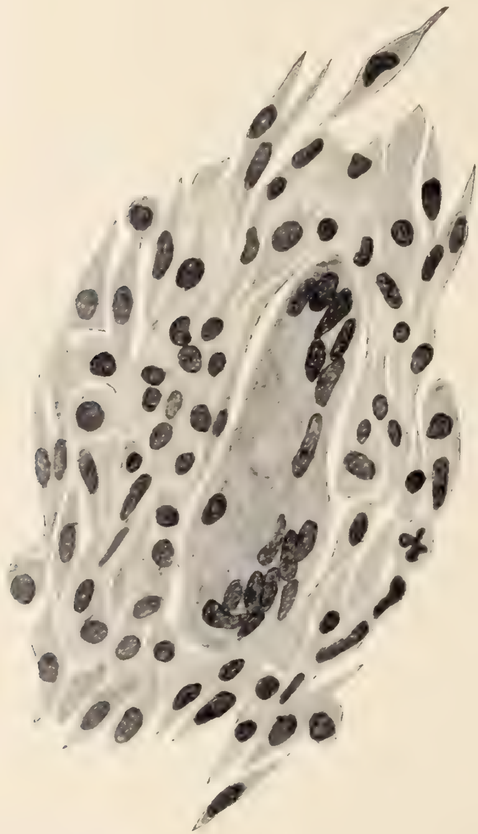


FIG. 1.—Camera lucida drawing of section from secondary nodule. Number 4 eyepiece and number 6 objective, Leitz.

home at Smithtown Branch, N.Y., and the other was the case of the small girl who had playfully bandaged a sore on her finger with cloths that had been contaminated with the pus from a sporothrix infection on the arm of another person (Case 33).

It has been suggested by Sutton and by Hyde and Davis that

the infection in man may be contracted from horses. There is, however, no direct evidence to support this statement. Hyde's patient had lived in a neighborhood where a number of horses had been suffering from a disease which was thought might have been sporotrichosis. I am informed, however, by several veterinarians

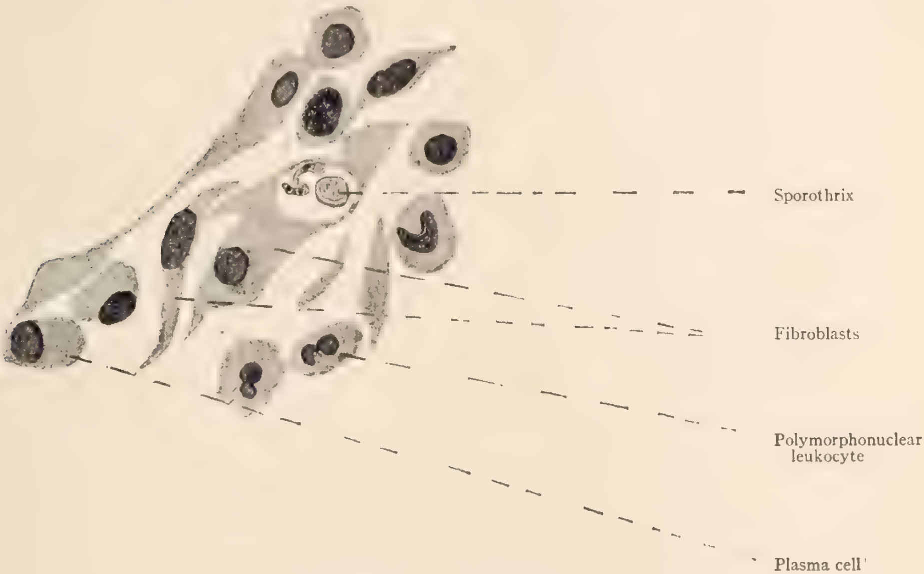


FIG. 2.—Showing sporothrix in the tissue of a secondary nodule. Camera lucida drawing: number 4 eyepiece and $\frac{1}{12}$ inch oil immersion objective, Leitz.

of this state who saw these horses that the horses were suffering from glanders and not from sporotrichosis.

HISTOLOGY OF THE SECONDARY NODULES.

Not a great deal is to be found in the American literature regarding the histology of the secondary nodules. I have recently had an opportunity to study several of the nodules, which were excised for me by Dr. E. P. Quain. The sections show that the nodules in the early stages are made up of embryonal connective tissue, which is infiltrated with plasma cells and a small number of polymorphonuclear leukocytes. The polymorphonuclear leukocytes are relatively few in number, whereas the plasma cells are abundant in some areas and more scarce in others. Relatively

large giants cell are frequently seen (Fig. 1). There seems to be no proliferation of capillaries.

In the later stages evidence of necrosis makes its appearance. This is indicated by a blurring of the section due to karyolysis and karyorhexis. The nuclei which still take on the hematoxylin stain are very often greatly elongated and distorted. Some of these areas cannot be distinguished from areas of tuberculosis. Finally liquefaction takes place and there may be a secondary infection with staphylococcus, resulting in the formation of pus.

Some writers make the statement that the sporothrix cannot be found in the secondary nodules. This evidently is a mistake, because it is very easy to isolate it in pure culture from these nodules, by carefully incising one, even before liquefaction has set in. I have isolated the organism in this manner from three cases. It is, however, not easy to find the organism in sections of the tissue from the nodules, but if a prolonged search is made they will be found. Fig. 2 is a camera lucida drawing showing the organism in the tissue from a secondary nodule.

A SIMPLE METHOD FOR ISOLATING ANAEROBES IN PURE CULTURE.*

J. P. SIMONDS AND A. I. KENDALL.

The difficulty of isolating anaerobes in pure culture has always been a serious handicap to the study of these organisms. The following method has proved so simple and easy of application that its publication seemed warranted.

Sixteen-ounce, French square, wide-mouthed bottles are plugged with cotton and sterilized by dry heat. With the bottles lying on their sides, sufficient blood agar is poured in to form a layer 2 to 5 mm. in thickness, and allowed to harden. As soon as the agar has hardened, the bottles should be turned on the opposite side, thus bringing the medium uppermost and preventing the water of condensation from running down on it.

If sterile blood for blood agar plates is not available Dorset's egg medium to which 1 per cent of dextrose has been added may be used. The medium is run into the bottles which must be kept lying on their sides, coagulated, and sterilized in the same way as Dorset's medium. By bringing up the temperature very slowly, the sterilization may all be done at once by heating in the autoclave for 15 minutes under 10 pounds' pressure. As the colonies are difficult to see on this yellowish-white medium, this difficulty should be obviated by the addition of litmus to the medium before sterilization. Under the anaerobic conditions produced in the bottle after inoculation, the litmus is more or less completely bleached. After the readmission of air, however, the color soon returns and assists very materially, not only by rendering the colonies more easily visible, but also by differentiating acid producers.

Our work with this method has been concerned chiefly with the isolation of anaerobes, especially *B. aerogenes capsulatus*, from stools but has proved equally applicable to material from various other sources. Tubes of milk from which the cream has not been removed are boiled for several minutes to drive off all dissolved

* Received for publication July 5, 1912.

air. These are then cooled and inoculated with the stool or other material being studied, and heated at 80° C. for 20 minutes. After 24 hours' incubation those tubes which give a characteristic reaction for *B. aerogenes capsulatus* are set aside for "plating."

Dilutions are made from these milk cultures by shaking up several drops, drawn from the bottom of the tube, in sterile water. Bent glass rods dipped in this dilution are then rubbed over the surface of the medium in the bottles, and closely fitting one-holed rubber stoppers carrying a closely fitting glass tube four inches in length are inserted in the mouths of the bottles. The outer end of the tube projects three-fourths of an inch beyond the stopper and carries a tightly fitting rubber tube three inches in length. The inner one inch of the tube is bent at an angle of 45° and the stopper turned so that the end of the tube points toward the side of the bottle opposite the medium.

As much air as possible is aspirated from the bottle and the rubber tube closed with a pinch-cock. The bottle is now placed on its side with medium uppermost and with a pipette 10 c.c. each of a 50 per cent solution of pyrogallic acid and 10 per cent solution of sodium hydroxid are run in through the rubber tube, care being taken to avoid letting air into the bottle. A few cubic centimeters of clean water are allowed to run in, with the same precautions, to free the rubber tube of alkali. Rubber gloves should be worn while introducing the fluids in order to avoid staining the hands. The efficiency of the apparatus and the absence of leaks are indicated by the presence of fluid in the glass tube and by the collapsed condition of the rubber tube behind the pinch-cock. Both these conditions are present if the apparatus is working properly. The bottle must be kept constantly on its side with the medium uppermost.

After incubation for 24 hours, the pinch-cock is carefully opened and the air allowed to flow in very gently to avoid spattering the fluid up on the medium. The stopper is then removed and the fluid poured out. Water is run in carefully and all the black fluid washed out. Care must be taken not to splash any water up on the surface of the medium. The bottles are now stood on end, mouth down, for 15 or 20 minutes to drain. If the litmus-

dextrose-egg medium is used the color will usually return to the litmus while the bottles are draining.

After the bottles have drained, colonies are fished and planted on slants of dextrose blood agar, dextrose agar, or dextrose-egg-medium. These are then inverted, without plugs, in a wide-mouthed jar (a pint fruit jar serves well) containing 5 to 10 gms. of dry pyrogallic acid. When the jar is nearly full of tubes water is poured in to a depth of three-fourths of an inch. As soon as the pyrogallic acid is completely dissolved the solution is covered with a layer of paraffin oil and 20 c.c. of a 10 per cent solution of sodium hydroxid is introduced below the oil by means of a pipette. The tubes are then incubated. This method is essentially that described by Rickards.[1] These slant cultures will usually be found to be pure, and are ready for use after 24 hours' incubation.

The method is simple, easy of application, makes possible the obtaining of pure cultures in the shortest possible time, and has proved very efficient and useful in our hands.

[1] *Centralbl. f. Bakt.*, I Abt., O. 1904, 36, p. 557.

EXPERIMENTAL INFECTIOUS ENDOCARDITIS.*

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The factors which determine infection of the endocardium are still quite obscure. A satisfactory explanation, based on experimental evidence, has not yet been offered as to why the valves are involved so much more frequently than the mural endocardium. While it is generally assumed that the bacteria localize by implantation, the greater frequency of mitral and tricuspid than semilunar endocarditis remains to be explained.

Köster[1] demonstrated that extension in an established human endocarditis may be due to embolism and suggested that endocarditis may begin as an embolic process. Experimental proof of this mode of origin is still lacking, however. Lissauer,[2] after an extensive review of the literature, concludes that while endocarditis may occur exceptionally as an embolic process, no one has been able to show experimentally that this mode of origin really plays a rôle in the localization of bacteria on the heart valves.

The great difficulty of producing endocarditis experimentally without previous injury to the valves is well known. Ribbert[3] produced endocarditis in rabbits by intravenous injections of emulsions of staphylococci and potato particles, and showed that the endocarditis was due, at least in some cases, to implantation. Orth and Wyssowitsch,[4] and Fulci[5] had similar results from injections of suspensions of staphylococci and also of streptococci with finely pulverized charcoal. These authors were not able, however, to produce endocarditis by intravenous injection without the foreign particles. Recently Lissauer obtained endocarditis in two of 20 rabbits after repeated intravenous injections of a non-virulent, white staphylococcus. One rabbit died in two months after six injections, the other in six months after 10 injections, both showing

* Received for publication July 12, 1912.

1 *Virchow's Arch.*, 1878, 72, p. 257.

2 *Cent. f. Allg. Path.*, etc., 1912, 23, p. 243.

3 *Fortschritte der Medicin*, 1886, 4, p. 1.

4 *Virchow's Arch.*, 1886, 103, pp. 300 and 333.

5 *Beiträge z. Path. Anat.*, 1908, 44, p. 349.

small vegetations on the mitral and tricuspid valves. He offers no explanation as to why repeated injections over a long period should produce endocarditis.

Horder[1] has shown that the cocci cultivated from the blood in cases of chronic infectious endocarditis as well as allied streptococci cultivated from the throat and feces are quite prone to produce endocarditis in animals. Other observers have had similar results. In previous papers I[2] have shown that the production of endocarditis by intravenous injections of organisms isolated from cases of endocarditis is due to peculiar qualities of the bacteria. It is noteworthy that the form of endocarditis in question, namely, a chronic or subacute form which begins insidiously on a previously diseased valve, often with no demonstrable source of infection, and which nearly always runs a fatal course, is due to bacteria of very low virulence. The various strains differ quite markedly in certain details, but at certain stages they all are freely susceptible to phagocytosis, adhere more or less markedly to the surface of solid media, grow in clumps in broth, and produce endocarditis quite regularly when injected intravenously in rabbits. When injected in other regions they produce only slight illness and are rapidly destroyed by phagocytosis.

Certain experiments will now be described, the results of which seem to throw light on some of the problems connected with this form of endocarditis.

1. *Experiments with pure cultures of cocci isolated from cases of chronic infectious endocarditis.*—Practically all injections were made into the marginal ear vein of half-grown rabbits. Five strains of the cocci used were isolated from the blood and two from the tonsils of cases of subacute or chronic infectious endocarditis. All produced a variable amount of green on blood agar plates, all fermented inulin at one time or another, and at the time of the experiments the growths adhered to the surface of blood agar and formed clumps in broth. All the strains were freely susceptible to phagocytosis and non-virulent in the usual sense. Suppuration and diffuse peritonitis could not be produced. The

[1] *Quart. Jour. of Med.*, 1909, 2, p. 289.

[2] *Jour. Infect. Dis.*, 1909, 6, p. 245, and 1910, 7, pp. 411 and 429.

cocci injected were usually grown both on blood agar and in ascites-dextrose-broth; in the latter case, the cocci were removed and suspended in suitable amounts of salt solution. The dose, relatively speaking, in most instances was exceedingly large, so large that the amount of the injected material was often sufficient to kill in 24 hours.

In Table 1 are given the essential facts that illustrate the results obtained. We see that the localization on the valves is an embolic process and not due to implantation. The clumps of bacteria lodge in the capillaries, produce hemorrhage, and probably because of the relatively slight vascularity in the valves the organisms grow into clumps before leukocytes are able to cause their destruction. Hemorrhages and endocarditis occur at the apex of papillary muscles in the same way. Hemorrhages occur at the base of the semilunar valves also, and vegetations have been seen to grow from this region (Plate 1, Figs. 4 and 5). In the rabbits which died or were killed soon after the injection, hemorrhages were observed in all experiments as follows: tricuspid valve, 28 times; mitral, 9 times; base of aortic semilunar valve, 4 times; base of pulmonary semilunar valve, 3 times; in papillary muscles (chiefly at apex), 20 times; kidney, usually glomerular, 11 times; subendocardial (other than papillary or valvular), 4 times; lungs, 7 times. General subserous hemorrhages were seen 6 times. Valvular hemorrhages occurred repeatedly without hemorrhages elsewhere. This was especially true if the dose was not sufficient to kill the animal in 24 to 48 hours.

On the injection of doses so large that the animal would die in a few minutes marked cardiac, renal, and pulmonary hemorrhages would result, but in no such instance were valvular hemorrhages observed. A certain time must elapse before the valvular hemorrhage takes place.

After having established the close relation between hemorrhage and endocarditis in the rabbit, I made a similar study in guinea-pigs. Valvular hemorrhages were observed only twice and endocarditis failed to develop, due no doubt to the absence of blood supply in the valves of this animal.

To emphasize further the great affinity of these cocci for the

TABLE 1.

Experiments with Cocci from Chronic or Subacute Infectious Endocarditis in Man.

Number of Rabbit	Intravenous Injections*	Time of Death after First Inoculation	Postmortem Examination
203.......	One very large dose of strain 1	24 hours	Hemorrhage at base of semilunar cusp (Plate 1, Fig. 4), and at apex of two papillary muscles in left ventricle, also in kidney, chiefly in glomeruli. Myocardium, gray; fatty degeneration of liver. Heart's blood, spleen, and surface of valve sterile. From hemorrhagic areas, pure cultures of organism injected.
221.......	One very large dose of strain 632	24 hours	Four hemorrhages in tricuspid valve, three along line of closure, the other, which is larger, is nearer base but extends to within 2 mm. of the free margin (Plate 1, Fig. 1). Two small hemorrhages in mitral valve. No hemorrhages anywhere else. Myocardium, gray and flabby. Right ventricle, dilated.
255.......	One large dose of strain B	48 hours	Four small hemorrhages of tricuspid valve and base of middle aortic semilunar cusp. Localized endocarditis of tricuspid valve and apex of papillary muscle. Four small vegetations are seen growing out of areas of hemorrhage.
191.......	Three medium-sized injections of strain 632 before and after animal passage	10 days	Vegetative tricuspid and mural endocarditis: thrombophlebitis of coronary vein; pulmonary infarction; peribronchial lymphadinitis. Original organism from vegetation and blood; typical encapsulated pneumococci from lymph glands and lung.
226.......	Two large doses of strain 632	9 days	Killed by ether. Vegetative endocarditis of tricuspid valve and apex of papillary muscle of left ventricle, cultures of which are positive. Blood, sterile. Ulceration along line of closure.
185.......	Three very large doses of strain E after four animal passages	14 days	Vegetative mitral and aortic endocarditis. Cultures yield organism injected.
189.......	One very large dose of strain E after seven animal passages	7 days	Vegetative tricuspid endocarditis; acute splenitis. Organism isolated resembles one injected.
182.......	Two medium-sized doses of strain E after three animal passages	10 days	Mitral and tricuspid endocarditis. Localized peritonitis. Organism isolated like one injected.
240.......	Two large doses of strain 657	22 days	Vegetative tricuspid, mitral, and mural endocarditis. The vegetation of the tricuspid orifice so large as to cause death by obstruction. The mural endocarditis involves the apices of the papillary muscles. The origin of these areas and the smaller vegetations are clearly subendothelial. The organism isolated from vegetation resembles closely the one injected.
237.......	One large dose of mixture of 10 strains, cultivated on blood agar for from three months to eight years	11 days	Mural endocarditis at apex of capillary muscle of right ventricle. Blood and joint fluids, sterile.

TABLE 1.—*Continued.*

EXPERIMENTS WITH COCCI FROM CHRONIC OR SUBACUTE INFECTIOUS ENDOCARDITIS IN MAN.

Number of Rabbit	Intravenous Injections	Time of Death after First Inoculation	Postmortem Examination
197	One very large dose of strain E after nine animal passages	8 days	Tricuspid, mitral, and mural endocarditis; miliary infarct of kidney; multiple pulmonary infarction, and pneumonia. Blood and spleen, sterile; vegetation and infarct in kidney yield coccus injected, while lung yields typical pneumococci.
188	Four large doses of strain E after seven and eight animal passages	8 days	Tricuspid and mural endocarditis; bronchopneumonia. Cultures from heart's blood negative; vegetation yields mostly cocci like the original and without virulence for guinea-pigs; lung and bone marrow yield pure cultures of an encapsulated, green-producing, inulin-fermenting diplococcus which kills guinea-pigs and rabbits.
223	One very large dose of strain E after eight animal passages	20 days	Massive vegetative endocarditis of tricuspid valve. Edema, hydroperitoneum, hydropericardium, and hydrothorax. Organism from vegetation resembles the one injected; pleural fluid gives pure culture of lanceolate, encapsulated diplococcus.
184	Two large doses of strain E after eight and nine animal passages	11 days after the first and one day after second injection	Pneumococcemia, acute splenitis; spleen yields typical pneumococci which kill rabbit in 24 hours from pneumococcemia.
209	One large dose of strain E after 10 animal passages	7 days	Killed. Healing mural and tricuspid endocarditis. Heart's blood, sterile. Crushed healing vegetation yields a few colonies of organism resembling the one injected.
208	Two large doses of strain E after 10 animal passages	24 days	Killed. Healing mural endocarditis of tip of papillary muscle of left ventricle. Heart's blood and healing area, sterile.
192	Two large doses of strain 632	50 days after first and one day after second injection	Healed localized endocarditis of mitral valve (see Plate 1, Fig. 6).
198	One very large dose of strain 632 after heating to 60° C. for one hour	24 hours	Hemorrhages in tricuspid and papillary muscle; cultures negative.
202	Three very large doses of strain 632 heated to 60° C. for one hour	17 days	Killed. Number of flattened, puckered, grayish areas in mitral valve, that is, healed endocarditis.

endocardium, the results obtained with two strains may be given. Strain 632 was injected intravenously in 32 rabbits; valvular hemorrhages were obtained 19 times; vegetative endocarditis, 6 times; healing or healed endocarditis, twice; pneumonia and endocarditis, 3 times; pneumonia alone, 3 times; and no gross lesions, 3 times. Strain E was injected into 22 rabbits; valvular hemorrhages were obtained 7 times; vegetative endocarditis, 6 times; healing or healed endocarditis, twice; pneumonia and endocarditis, twice; pneumonia only, 3 times; and no lesions, twice.

Hemorrhages and scars of the valves have been produced by the injection of cocci killed by heating to 60° C. for one hour. In five instances unmistakable evidence of healing or healed endocarditis was obtained (see Plate 1, Fig. 6). Three of these were found in the tricuspid and two in the mitral valve.

Microscopic sections of valves and papillary muscles, the seat of hemorrhages, show dilated blood vessels, desquamation of endothelial cells, and subendothelial and muscular extravasation of blood. What appears to the naked eye as subendothelial hemorrhages in valves and papillary muscles always proves to be such on microscopic examination (see Plate 2, Fig. 7). Bacterial emboli are not found in the areas of hemorrhage and the adjacent capillaries within 24 hours after the injections; at the end of 48 hours, however, bacterial masses are easily found, usually adjacent to an area of hemorrhage (see Plate 2, Fig. 8). At this time there is hardly any leukocytic infiltration about the bacterial masses, which is in marked contrast to the lesions in the glomeruli of the kidney in which leukocytic infiltration is marked at the end of 48 hours. In no instance is there any evidence of thrombosis at this early stage nor evidence that leukocytes were the carriers of bacterial clumps.

The lesions of the kidney in this experimental endocarditis in the rabbit are not unlike those described first by Aschoff[1] and Gaskell,[2] and more recently by Baehr,[3] in ulcerative or chronic infectious endocarditis in man. They consist essentially of glomerular hemorrhages due to bacterial emboli followed by leukocytic infiltration and later almost invariably by sclerosis. In two old rabbits, however, which were injected repeatedly and in which no endocarditis developed, some of these areas, instead of healing promptly, became the seat of minute, grayish-white masses made up of bacterial clumps, leukocytes, etc., similar to the endocardial vegetations. The hemorrhages occur almost exclusively in the fine capillaries of the glomerular tuft. At the end of 48 hours leukocytic infiltration may extend beyond Bowman's capsule. At the time of death from endocarditis in the rabbit fresh renal hemorrhages are rare, but healing or fibrous glomeruli are commonly observed. Red blood corpuscles have been found in the urine.

[1] *Pathol. Anat.*, Jena, 1911.

[2] *Jour. of Path. and Bact.*, 1912, 16, p. 283.

[3] *Jour. Exper. Med.*, 1912, 15, p. 330.

Table 1 shows an interesting fact which is in harmony with results obtained previously. After repeated animal passage these strains of cocci change into pneumococci, both as regards form and pathogenic power. As virulence increases, clump formation disappears, they no longer adhere to solid media, and only rarely produce endocarditis, but, instead, cause death from bacteriemia or pneumonia. Fatal pneumococcemia without localization was observed 11 times; bronchopneumonia, 7 times; and lobar pneumonia, 5 times. The strains isolated from the animals with pneumonia on further animal passage produced rapidly fatal pneumococcemia and not pneumonia. Pneumonia and endocarditis were observed in the same animals 5 times. The organisms isolated from the lung and peribronchial lymph glands in each instance resembled typical pneumococci while those from the depths of vegetations resembled closely the endocarditis strains. One experiment will serve to illustrate how the results were obtained: Strain E was easily differentiated from typical pneumococci by means of blood agar plates; it produced grayish-white colonies which adhered moderately, and which were surrounded by a very narrow zone of green. The strain used was grown from a single organism isolated for me by Mr. Moon by the Barber method. A medium-sized rabbit was given two intravenous injections, five days apart. The strain was then rapidly passed through a series of nine animals. A moderately sized dose was injected, the cocci now appearing to be typical pneumococci. The rabbit died in four days with vegetative aortic endocarditis and lobar pneumonia. The blood after death was sterile. From the lung, peribronchial lymph glands, and spleen pulp, pure cultures of typical pneumococci were isolated. From the depths of the vegetation the original endocarditis coccus was obtained in pure culture and from the superficial layers of the vegetation, both varieties.

In this connection I may state that Dr. Dochez, of the Rockefeller Institute for Medical Research, to whom I am indebted for a subculture of strain E, noted a number of months previously a similar reversion to the characteristics of typical pneumococci as virulence was restored by animal passage.

Lobar or bronchopneumonia was produced with each of the

strains as the virulence attained a certain point through animal passage. This result corresponds with the observations of Wadsworth[1] who found that in order to produce pneumonia in rabbits with pneumococci, a properly balanced relation between virulence and resistance of the host is necessary. Pneumonia was especially apt to occur when the rabbit had been injected once or twice previously. This suggests the idea that sensitization probably played a rôle in the development of the pneumonia.

A series of guinea-pigs were injected with heated cocci of a strain obtained from endocarditis. Two weeks later they were injected intravenously with equal doses of heat-killed and living cocci of (1) the strain as obtained from the blood, (2) the same strain after it had been converted into a pneumococcus by animal passage, and (3) with a strain of virulent pneumococcus from the blood of a case of pneumonia. All the animals reacted with symptoms of immediate anaphylaxis. Those receiving the live cultures reacted more violently than those receiving the heat-killed cocci. The bacteria disappeared from the blood more rapidly in the sensitized than in the non-sensitized pigs. This result at once suggests the idea that because the strains from endocarditis sensitize guinea-pigs to typical pneumococci they must themselves be pneumococci. This, however, cannot be regarded as settling the question conclusively because I[2] have shown that the proteins of the streptococcus and the pneumococcus are so nearly alike as to sensitize for each other.

The marked change which is brought about in these organisms by animal passage and cultivation under varying conditions led to certain further studies. Six strains were selected. In order to meet the objection which might be raised that I was dealing with mixed cultures in my previous work—which, however, is unlikely—the growths used were obtained from single bacteria of each of the strains isolated by Mr. Moon by a modification of the Barber method. Four of the strains were obtained from the blood during life and one from the tonsil in cases of chronic infectious endocarditis; one strain came from the pus of an empyema where it was associated with *B. fusiformis*. They all resembled closely Schott-

[1] *Am. Jour. Med. Sci.*, 1904, cxxvii, p. 851.
[2] *Jour. Infect. Dis.*, 1911, 9, p. 190.

müller's *Streptococcus viridans*. The four strains from the blood adhered more to the surface and produced less green. All the strains, grown in each instance from single bacteria, were made to take on the morphological, cultural, and pathogenic features of typical pneumococci. More animal passages were necessary with the ones obtained from the blood than with those from the throat and pus before changing to typical pneumococci. Each of 16 strains which I have studied in this way, obtained mostly from the blood in cases of endocarditis, though also from the throat and elsewhere, have been changed into pneumococci in this way. I know that the idea that these strains from endocarditis are modified pneumococci is not generally held, but in view of the facts cited, the conclusion is forced on me that the organisms which are isolated from this type of endocarditis, the organism designated by Schottmüller as *Streptococcus viridans* and by Horder as "saprophytic streptococci," are in reality pneumococci that have become attenuated and peculiarly modified as the result of environmental conditions. For this reason I doubt the advisability of applying a special name, such as "Endocardococcus," to these organisms as suggested by Libman. The various strains also differ quite markedly in important respects. Certain strains of staphylococci, and probably also of influenza bacilli, from endocarditis likewise show this affinity for the endocardium and tendency to clump formation, both these qualities being lost simultaneously.

2. *Experiments with cultures of cocci isolated from chronic infectious endocarditis mixed with cultures of streptococci.*—Fourteen medium-sized rabbits were injected intravenously with mixtures of endocarditis cocci and streptococci in order to observe the results (Table 2). The animals receiving the largest quantities of endocarditis cocci received the smallest quantities of streptococci and vice versa. The differentiation of these bacteria is easy on the blood agar plate. The organisms from endocarditis produce a small colony surrounded by a green area, the streptococcus a small colony surrounded by a wide clear zone of hemolysis. Hemorrhages in valves and papillary muscles which resembled in appearance, size, and location those following intravenous injection of pure cultures of the endocarditis strains were observed in all but one animal.

This latter animal died soon after injection. The hemorrhages in the valves seems to serve as a protection because the organisms lived longer here than elsewhere. In no instance were endocarditis

TABLE 2.

EXPERIMENTS WITH MIXTURES OF ORGANISMS FROM ENDOCARDITIS AND WITH STREPTOCOCCI.

Number of Rabbit	Strain and Size of Intravenous Injections		Time of Death after Inoculation	Postmortem Examination
	Organism 632 from Endocarditis	Streptococcus		
239	Very large	$\frac{1}{4}$ of blood agar slant	24 hours	Small pulmonary hemorrhages; hemorrhage of tricuspid valve and apex of one papillary muscle in left heart. No other visible hemorrhages. Blood, spleen, and joint fluids gave hemolyzing colonies only. Crushed portion of hemorrhage in valve and papillary muscle, chiefly hemolyzing but also some green colonies.
233	Medium	$\frac{1}{100}$ of blood agar slant	24 hours	Multiple small and one large hemorrhage in leaflet of tricuspid valve. No other hemorrhages. Myocardium gray, flabby. Blood, sterile; knee joint, pure hemolyzing colonies; spleen, equal number of hemolyzing and green-producing colonies; washed valve, sterile; crushed valve, green-producing colonies only.
P 872	Medium	$\frac{1}{100}$ of blood agar slant	48 hours	Hemorrhages in leaflet of mitral and tricuspid valves, and apex of papillary muscle. Blood and spleen sterile; joints, small number of hemolyzing colonies; washed valve, sterile; crushed area of hemorrhage, 10 green-producing colonies.
234	Medium	$\frac{1}{10}$ of blood agar slant	18 hours	Small hemorrhages in tricuspid valve. No other hemorrhages. Heart muscle, flabby, gray. Blood, very dark and clotted. Blood and spleen gave three times as many hemolyzing as green-producing colonies; joint, hemolyzing colonies only.
229	Small	One blood agar slant	48 hours	Hemorrhages in tricuspid valve and at base of one pulmonary semilunar cusp. Serofibrinous peritonitis and pleuritis. Myocardium, flabby, gray. Cultures yielded hemolyzing colonies from everywhere except crushed valve from which a few green colonies also developed.
232	Medium	$\frac{1}{10}$ of blood agar slant	12 days	Vegetative mural endocarditis at apex of papillary muscle right ventricle; arthritis right knee joint. Blood and spleen few, joint very many hemolyzing colonies. Surface of vegetation, chiefly hemolyzing colonies, from depth, green-producing colonies only.

cocci found in the joint fluids, and only four times in the blood and then in relatively small numbers. The streptococcus used was obtained from the throat in a case of tonsilitis. It had been cultivated on blood agar for three months and was only moderately

virulent. In the injected rabbits it was found usually in the blood and spleen and in every instance in the joints. Two rabbits in which the dose of endocarditis cocci was small developed arthritis without endocarditis. The hemolytic streptococcus was present in the joints in pure form early in the attack while later cultures were repeatedly negative even though the arthritis persisted for weeks. One rabbit (232, Table 2) developed both endocarditis and arthritis and the cultures here showed that the endocarditis coccus was responsible for the endocarditis and the streptococcus for the arthritis.

Five guinea-pigs, weighing from 200 to 250 gms., were also injected. One showed valvular hemorrhages, two developed arthritis, but none endocarditis. The hemolytic streptococcus was demonstrated soon after injection in large numbers in the joint fluids, while the endocarditis coccus was not found in any.

The results obtained furnish additional evidence to the effect that the arthritis in acute articular rheumatism may be due to hemolytic streptococci and suggest that the endocarditis may be due to the organisms which commonly produce endocarditis. It is possible, however, that in rheumatism streptococci are concerned which are intermediate between these two groups and which may produce both endocarditis and arthritis. Indeed the work of Poynton and Payne[1] and others points strongly in that direction.

3. *Experiments with mixed aerobic and anaerobic cultures of cocci from the throat.*—A series of experiments was made by injecting rabbits intravenously with mixed aerobic and anaerobic throat cultures grown on blood agar slants. The cultures were obtained from normal tonsils and from the tonsil of a case recovering from an attack of Vincent's angina. The aerobic cultures contained the green-producing, clump-forming cocci in predominating numbers ("Streptococcus viridans"), the micrococcus catarrhalis, and staphylococcus in large numbers, the micrococcus tetragenus, and a gram-negative bacillus; the cultures from the case of Vincent's angina contained in addition a few colonies of hemolytic streptococci. The anaerobic cultures contained the above strains and in addition the bacillus fusiformis. Three small rabbits were injected each

[1] *Lancet*, 1900, 2, p. 861.

with the aerobic cultures from four to six blood agar slants. Two died in 24 hours. Both showed valvular hemorrhages. The third died in six days from vegetative endocarditis of the tricuspid valve. The cultures of the blood and the surfaces of the valves in the first two animals showed a few staphylococci and the micrococcus catarrhalis, while from the crushed areas of hemorrhage there developed almost a pure culture of green-producing coccus; the cultures from the blood and vegetation of the rabbit which died of endocarditis developed this coccus in pure form. Four rabbits were injected with the anaerobic cultures, one with cultures from the normal throat and three with those from Vincent's angina. The former died of endocarditis at the end of seven days, cultures from vegetations and blood yielding a pure growth of the green-producing coccus. The other three also developed endocarditis. One rabbit (270) died in 72 hours. It showed fading hemorrhages in the valves and papillary muscle, and beginning vegetations. Cultures from the blood were sterile, while both anaerobic and aerobic cultures from the crushed valve yielded the green-producing coccus in pure form. A rabbit (265), which was injected three times in 10 days, died of a large thrombotic growth originating in the tricuspid valve. Smears and anaerobic cultures yielded both the bacillus fusiformis and the coccus. The third rabbit, (280) which was injected only once, was killed at the end of five days. A vegetative tricuspid and mural endocarditis was found. The cultures and smears gave the coccus in pure form.

4. *Experiments with mixed cultures of "Streptococcus viridans" and the bacillus fusiformis, and with the bacillus fusiformis only.*—The experiments showed that the hemorrhages found at the end of 48 hours as well as the endocarditis were due to the coccus and not to the bacillus fusiformis because the latter was absent in all the cultures and because neither hemorrhages nor endocarditis could be produced by injections of the latter in pure culture.

GENERAL CONSIDERATIONS AND CONCLUSIONS.

Endocarditis, caused by streptococci or pneumococci, and which develops in the course of a severe infection, runs a rapidly fatal course. Hence it does not seem likely that the simple or benign

forms of endocarditis, following streptococcus tonsilitis for example, or developing in the course of chorea or rheumatism can be due to highly virulent bacteria, but must be due rather to such cocci as produce endocarditis in rabbits. The same holds true for cases of unrecognized endocarditis in the young which later leads to valvular disease (nearly always mitral regurgitation with more or less stenosis). In the light of my experiments the sclerosis of the valves in such cases may be due either to repeated hemorrhages in the valves from bacterial emboli or, what is more likely, to a mild infection which gives no noteworthy clinical symptoms.

Endocarditis is more common in the young than in adults. In the light of my experiments the greater susceptibility of the endocardium in children may be explained as due to the presence of capillaries in the valves at this age. The attacks are relatively mild, blood cultures sterile, and recovery ensues, leaving a damaged valve. The very nature of these attacks makes it almost certain that the microorganisms in question are of a low grade of virulence. Now it is a well known fact that the chronic, malignant endocarditis under special consideration is ingrafted almost invariably on a previously diseased and sclerosed valve. My results suggest strongly that the same type of organisms can produce both the simple endocarditis and the fatal or malignant form when ingrafted on an old lesion. In the former case conditions for repair are better and healing results before the vegetations ulcerate. During the attack the valve becomes vascularized, but later scar formation ensues and the valve now becomes relatively avascular, contains exceedingly fine capillaries, and a condition is established which predisposes to reinfection. On account of the sclerosed condition of the valve, repair is more difficult, the vegetations grow large, ulceration takes place, and death eventually results. The fact that efforts at healing (sclerosis, calcification) even in the fatal cases in man are often present, as pointed out by Libman, together with the fact that I have produced simple endocarditis and sclerosis of valves experimentally, with cocci from chronic forms of endocarditis, speaks in favor of the view that, contrary to what is assumed on clinical ground, the organisms which produce the fatal form of the disease may also produce different grades of simple or benign endocardial inflammation.

The results of these experiments show that the endocarditis in the rabbit which follows injection of cocci which I believe to be modified pneumococci, is due to an embolic process. There is produced first valvular hemorrhage from which vegetations develop. The localization in the endocardium of the cocci from cases of chronic infectious endocarditis and of allied cocci from the throat, is due in large part to the presence of fine capillaries in the valves and to the peculiar mode of growth of the cocci. The relatively avascular structure of the valve serves to protect the cocci from leukocytes long enough to allow them to develop so as to produce the characteristic clumps around which fibrinous or other material is precipitated which for mechanical reasons again serves to protect the organisms. The production of valvular hemorrhages and of endocarditis by the simple intravenous injection of these cocci is an almost constant result so long as they form clumps and adhere to surfaces, but almost unattainable when this property has been lost either from artificial cultivation or animal passage.

It is necessary to inject intravenously an exceedingly large dose or smaller doses repeatedly in order to cause fatal endocarditis in rabbits. Otherwise healing results. Half-grown rabbits yield the best results, it being difficult to produce endocarditis at all in old rabbits, while in the very young, healing is more apt to occur.

The affinity of the endocarditis strains of cocci for the endocardium and of streptococci for joints is shown by the results of injections of mixtures of these organisms, and the exact cause of such affinity is unknown.

The injection of mixed aerobic and anaerobic cultures from the tonsils is followed by valvular hemorrhages and endocarditis, due to the presence of cocci similar to those found in chronic infectious endocarditis. The bacillus fusiformis does not seem to cause endocarditis in the rabbit.

The conclusion drawn in my earlier papers that the cocci in question are modified and attenuated pneumoccoci has received additional support because growths from single cocci from strains isolated originally from the blood of cases of endocarditis as well as from throats, when passed through animals, take on cultural and pathogenic properties indistinguishable from those of typical pneumococci.

My results also suggest strongly that the organisms belonging to this group may cause various forms of endocarditis designated as "simple infective endocarditis" in man as well as the malignant form of chronic infectious endocarditis.

EXPLANATION OF PLATES.

PLATE 1.

FIG. 1.—Photograph of a heart showing three hemorrhages in tricuspid valve of rabbit 221 (see Table 1). Portion showing the largest hemorrhage was cut out and sectioned (see Plate 2, Fig. 8).

FIG. 2.—Hemorrhages into tricuspid valve. Death 24 hours after a very large intravenous injection of strain 632.

FIG. 3.—Tricuspid and mural endocarditis. Ulceration has taken place on the side of closure. Death nine days after a large intravenous injection of strain E.

FIG. 4.—Subendothelial hemorrhage at base of semilunar cusp of pulmonary valve.

FIG. 5.—Vegetative endocarditis of aortic valve growing from base of cusps.

FIG. 6.—Healed, localized endocarditis of mitral valve, showing scars, thickenings, and some puckering of valve leaflet.

PLATE 2.

FIG. 7.—Subendothelial hemorrhages of tricuspid valve; death 24 hours after intravenous injection. Note the marked extravasation of blood, absence of leukocytic infiltration, and the intact endothelium on both surfaces of the valve. Hematoxylin and eosin. 250 diameters.

FIG. 8.—Photomicrograph of an early vegetation in the tricuspid valve, 48 hours after an intravenous injection. Note the many diplococci to the right of a small hemorrhage between the endothelial cells, and the absence of leukocytes. 800 diameters.

FIG. 9.—Photomicrograph of vegetation of tricuspid valve nine days after intravenous injection. The round masses at bottom are bacteria surrounded by a wide zone of leukocytic infiltration. Endothelium still intact, but encroached upon. Ulceration has not yet taken place. 120 diameters.

PLATE I.

FIG. 1

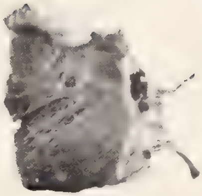

FIG. 2.

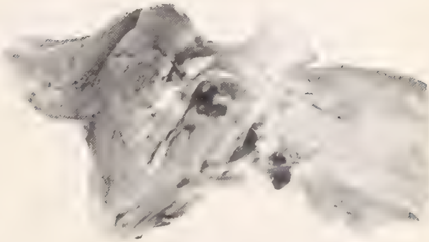

FIG. 3.

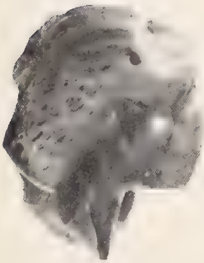

FIG. 4.

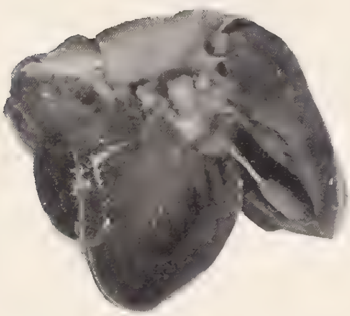

FIG. 5.

FIG. 6.

PLATE 2.

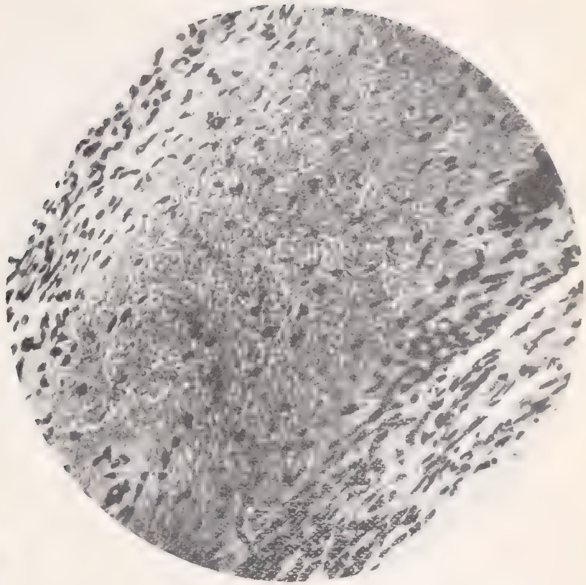

FIG. 7.

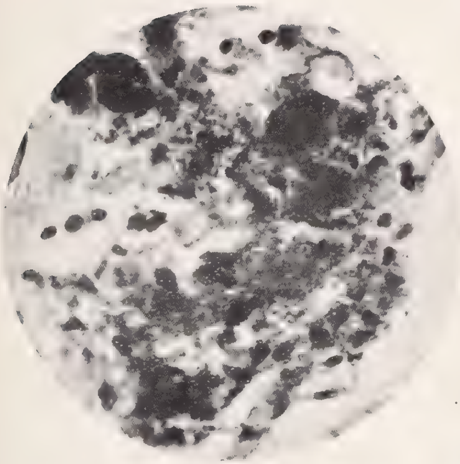

FIG. 8.

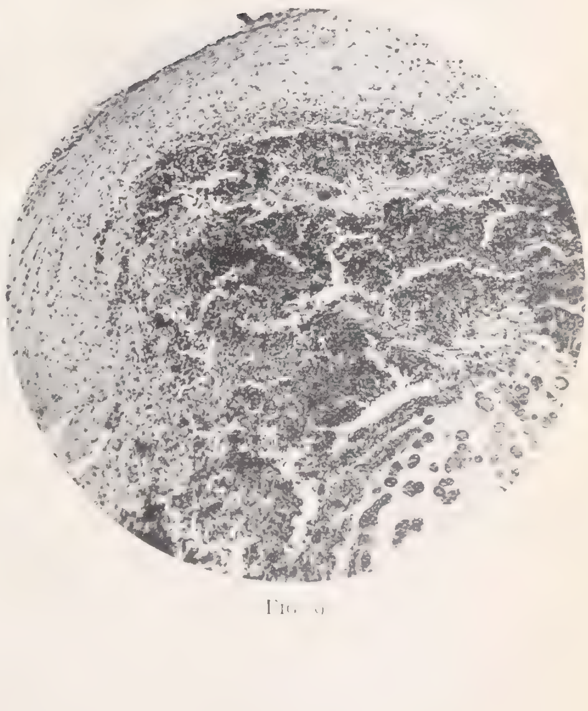

FIG. 9.

VARIATIONS IN THE COMPLEMENT CONTENT OF SERUM AND PLASMA.*†

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That doubt as to the presence or absence of complement in the circulating plasma should still exist after several years of observation and repeated experiment, seems, at first sight, somewhat extraordinary. That there is a difference of opinion on the subject is evident from the diverse statements which, in turn, are based on the observation of experiment, published by different authors. Gengou,[1] over 10 years ago, satisfied at least himself that plasma does not contain a complementary body capable of reactivating specific sera *in vitro*. Since this time a moderate number of contributions have appeared relative to the subject; a relatively small proportion of these authors substantiating Gengou's observations, the majority stating their belief in the erroneousness of his conclusions.

The most recent article upon this subject is that by Addis,[2] who, basing his opinions on the results of his experiments upon cock's plasma and serum, states unqualifiedly that complement is present in substantially equal quantities in both plasma and fresh serum. He refers briefly to the work of Hermann, Domery, Sweet, Hewlett, Lourt, Schwartz, and others, all of whom, with the exception of Hermann, have been unable to corroborate Gengou's findings.

The reasons for such diverse opinions are due to the fact that the methods adopted for the estimation of complement quantitatively and qualitatively are liable to afford possibilities of error which are of the greatest importance. The simplest and most

* Received for publication, July 11, 1912.

† Read by title before the American Association of Pathologists and Bacteriologists, Philadelphia, 1912.

‡ Aided by the John Baillie Fund for Immunity Research.

[1] *Ann. de l'Inst. Pasteur*, 1901, 15, p. 232.

[2] *Jour. Infect. Dis.*, 1912, 12, p. 208.

satisfactory means of estimating the complement content of serum and the one most commonly employed in routine experiment, as in the Bordet-Gengou reaction, is that which measures the activating property of a serum on amboceptor, especially as this is evidenced in the production of hemolysis of sensitized red blood cells. For general purposes such a method of determining the quantity of complementary body leaves little to be desired. If, however, we attempt to employ this method of measuring the complement contained in the fluid portion of uncoagulated blood, i.e., plasma, as has customarily been done, we find that a serious error may be encountered. This is due to the fact that it is probably impossible so to wash the erythrocytes used as the indicator that no other substance present in the blood from which they are obtained is present in the suspension.

It is quite possible, by means of the employment of paraffined tubes, albolene- and petrolatum-protected syringes and needles, and rapid chilling and centrifugalization, to procure a plasmatic fluid even from guinea-pigs' blood which is free from thrombokinase, or at least to limit its production to such minute proportions that fibrin formation will not occur even though the fluid be placed in contact with unprotected glass surfaces. This fact is well recognized by those experimenting on the cultivation of tissues *in vitro*. It is further noted by these observers that it is not necessary that leukocytes be added to the plasma in order that clotting may follow; simply the introduction of a small number of connective tissue cells into such a fluid is almost instantly followed by the deposition of fibrin in radiating lines from the point of application. Similarly we find that the addition of washed erythrocytes to fluid plasma is rapidly followed by the formation of a coagulum.

It appears, therefore, that sufficient thrombo-kinase to induce fibrin formation is present, closely associated with the cells, in erythrocyte suspensions such as are employed in complement-determining hemolytic reactions. If this be true, it appears possible that some body bearing a relationship to complement similar to that which thrombo-kinase bears to fibrin may likewise be present in such suspensions. I am of opinion that complement as it is

generally known and recognized in fresh serum does not exist as a staple constituent of normal plasma and I have attempted to prove this to be the case by means of the experiments described in this paper. I believe, furthermore, that the diverse opinions expressed by various investigators are explained chiefly at least by the experimental error just mentioned.

It will be noted that the evidence produced in the following pages, and on which this view is based, is both direct and indirect in character. Certain experiments appear to prove directly that complement is not present in plasma, while those which demonstrate the variations in the complement content in one and the same serum specimen if the latter be subjected to different conditions, especially in so far as this refers to temperature and time, should be looked upon as being almost equally suggestive.

In this paper I will confine myself to a study of the factors determining the development of complement, reserving the important question of the cause of the natural deterioration of serum in this respect for a later contribution.

TECHNIC.

The experiments upon which this paper is based were all performed with full-grown guinea-pigs, weighing from 450 to 550 gms. In procuring material the animals were anesthetized (with ether) and the blood withdrawn directly from the heart. Following removal, the blood was kept for varying lengths of time at different temperatures such as are mentioned in the text and tables. Rapid chilling, in those experiments demanding such treatment, was accomplished by means of exposure to a mixture of ice and common salt for several minutes, but not long enough to freeze the blood. Standard-sized centrifuge tubes were employed in all instances for receiving blood, quantities varying from 2.5 to 5.0 c.c. of blood being placed in each. This fact is noted, since the volume of blood exposed to variations in temperature is of importance inasmuch as smaller quantities are more rapidly affected by the temperature of the surrounding medium, water, air, etc. As a general rule from 3 to 5 c.c. were removed from each pig examined.

Immediately before use, all specimens were placed in the centrifuge and subjected to a speed of from 5,000 to 7,000 revolutions for from one to three minutes depending on the difficulty with which the serum was obtained clear.

In procuring plasma, paraffined centrifuge tubes placed in a freezing mixture were employed to receive the blood, which was obtained in a 5 c.c. all-glass syringe, using a large bore needle, the whole being heavily coated with vaseline.

The hemolytic series employed throughout has been the "sheep-rabbit" system, the amboceptor having in all cases an extremely high titer. Unless otherwise stated the total quantity of fluid employed in each experiment was 1.0 to 1.25 c.c., being made up with 0.85 per cent sodium chlorid solution. The suspension of sheep's erythrocytes employed was approximately 0.15 per cent, the mixtures being prepared in bulk so that the possible error which might arise from an unequal distribution of cells might be excluded.

INTERPRETATION OF TABLES.

The accompanying tables have been prepared in a more or less uniform manner; the horizontal line of figures stands for the corresponding numbers of drops of serum, which unless otherwise stated, have been diluted one to two, each drop having a value of approximately 1/40th c.c. The relative degree of hemolysis obtained is described by means of the employment of numerals instead of the customary collection of plus and minus signs. Complete hemolysis occurring during the time limit set for the experiment has been signified by the numeral 6; absence of hemolysis is shown by zero; while intervening stages of lysis have been designated by means of the intervening numerals.

EXPERIMENTS.

Tables 1, 2, and 3 give the results of observations on the available complement content of serum when the blood from which it is obtained is exposed to different degrees of temperature. It will be noted that the maximum amount of demonstrable complementary body is obtained in blood kept at body temperature, the minimum amount being found in blood at 0° C.

These experiments, in addition to drawing attention to the fact just mentioned, show that the amount of complement available for hemolytic purposes is somewhat greater even when the blood is kept under most favorable circumstances at the end of one hour than at the end of 15 minutes. It is worthy of note also that the amount of complement developed in the serum of different animals

TABLE 1.

ACTIVATING POWER OF GUINEA-PIGS' SERUM UNDER DIFFERENT CONDITIONS AS REGARDS TEMPERATURE.

		3*	6	7	8	10
Guinea-pig 1	37° 1 hr., 5° 23 hrs.	3	4	4	5	6
Guinea-pig 2	37° 1 hr., 5° 23 hrs.	3	(Broken)	4	6	6
Guinea-pig 1	0° 1 hr., 5° 23 hrs.	1.5	1.5	2	4	4.5
Guinea-pig 2	20° 1 hr., 5° 23 hrs.	1.5	1.5	2	4.5	6

*Serum diluted 1 to 2

TABLE 2.

ACTIVATING POWER OF GUINEA-PIGS' SERUM PRESERVED AT DIFFERENT TEMPERATURES.

		2	4	5	7	9
Guinea-pig 3	1 hr 0°	0	2	3	4.5	6
Guinea-pig 3	1 hr. 20°	0	4.5	5	6	6
Guinea-pig 3	1 hr. 37°	6	6	6	6	6
Guinea-pig 4	1 hr. 0°	0	2	3	4.5	6
Guinea-pig 4	1 hr. 20°	0	4.5	5	6	6
Guinea-pig 4	1 hr. 37°	5	6	.6	6	6
Guinea-pig 5	1 hr. 0°	0	3	4	6	6
Guinea-pig 5	1 hr. 37°	5	6	6	6	0

TABLE 3.

ACTIVATING POWER OF GUINEA-PIGS' SERUM PRESERVED AT DIFFERENT TEMPERATURES.

		2	3	4	5	7
Guinea-pig 6	37° 1 hr., 5° 18 hrs.	4.5	6	6	6	6
Guinea-pig 6	37° 15 min., 5° 18¾ hrs.	3	4.5	5	6	6
Guinea-pig 7	37° 1 hr., 5° 18 hrs.	4	6	6	6	6
Guinea-pig 7	37° 15 min., 5° 18¾ hrs.	2	4	5	6	6
Guinea-pig 6	5° 19 hrs.	1.5	3	4	5	6
Guinea-pig 7	5° 19 hrs.	2	3	4	5	6

is very nearly absolutely constant in amount. This fact I have sought to emphasize in a previous publication.[1] As might well be expected, it is found that although the development of complement proceeds very slowly at lower temperatures, the longer sera are stored in the ice-chest the more nearly does the complement content of different specimens tend to become equalized.

[1] *Jour. Infect. Dis.*, 1911, 8, p. 427.

The same feature of complement variation depending upon temperature is demonstrated by an experiment in which equal quantities of sera (four drops of a one-in-three dilution) from the same animal, but exposed to different temperatures, were tested with varying quantities of hemolytic body and a constant volume of erythrocytic suspension. By this time it was shown that serum preservation at a temperature of 37° for one hour rendered three drops of an amboceptor dilution sufficiently active, whereas serum procured from blood kept at 20° required the addition of eight drops of the same dilution of amboceptor containing serum to induce complete hemolysis.

While it has not been possible for the writer to prove the absolute absence of complement in plasmatic fluid from the guinea-pig, the following experiments, similar examples of which have been frequently repeated, were carried out, which prove that the amount developing can be markedly limited. It must be stated, however, that the various technical steps must be very carefully carried out. Otherwise, even the relative absence of complement, as evidenced by the accompanying experiments, cannot be demonstrated.

TABLE 4.

PROTOCOL SHOWING RELATIVE ACTIVATING POWER OF GUINEA-PIG SERUM AND PLASMA.

	2	4	5	6
Serum 1	3	5	6	6
Serum 2	3	6	6	6
Serum 3	3	5	6	6
Plasma 1	0	0	4	5
Plasma 2	0	0	4	5
Plasma 3	0	1	4	5

Serum prepared as follows: Clotted at 38° 15 minutes; chilled; centrifugalized.

Plasma procured as follows: Paraffin method, chilled, centrifugalized.

Total time of experiment, 20 minutes.

Serum and plasma used undiluted.

If the quantity of serum barely sufficient to bring about complete hemolysis under the conditions of the experiment, namely 1 c.c., be accepted as a unit, it will be noted that a similar quantity of plasma contains an amount of complement so small that no hemolysis whatever occurs.

The following series of experiments also demonstrate the relative absence of complement in blood preserved at a temperature below that of the body.

TABLE 5.

COMPARATIVE ACTIVATING POWER OF SERUM FROM THE SAME ANIMAL SUBJECTED TO DIFFERENT TEMPERATURES.

		3	5	6
Guinea-pig 15	Clotted 37° 1 hr.	6	6	6
Guinea-pig 15	Whipped 20° 35 min.	3	6	6
Guinea-pig 15	Whipped, chilled, 2 hours	2.5	4.5	6
Guinea-pig 15	Diluted with saline, 1 to 8, 0° 30 min.	1	2	0
Guinea-pig 15	Clotted 45° 1 hr.	6	6	6

Experiments were also employed in which coagulation was prevented by means of sodium citrate. Results seemed to show that, if anything, the development of the complementary body was hastened by this method, this result being due, it is assumed, to the action of the citrate upon the white blood cells.

In view of the circumstantial evidence in favor of the development of complement in the form in which it is usually considered, subsequent to the removal of the blood from the body, and in view of the difficulty experienced in proving by the direct method the absence of complement in plasma as obtained by ordinary methods, I have employed what must be termed the indirect method of demonstrating the presence of complement. I have already mentioned my reasons for believing that this method ought to be productive of more positive results. The experiments to be enumerated seem to justify this contention.

If the presence of complement in plasmatic mixtures, as demonstrated by myself and numerous other observers, be due, as I believe, to the interaction of some substance present in the erythrocyte mixture and a complementogenic body in the blood fluid, it appeared to me that it should be possible to maintain this complementogenic substance intact if the blood fluid were exposed to the action of a specific antigen and in the absence of hemocellular products. For this purpose, guinea-pig plasma was mixed with an excess of *B. typhosus* antigen and antityphoid amboceptor derived from an immune rabbit. Following incubation for one

hour there was added to the mixture a quantity of "anti-sheep-rabbit" serum and washed sheep's erythrocytes, with results as detailed in Table 6.

TABLE 6.

BORDET-GENGOU REACTIONS EMPLOYING GUINEA-PIG SERUM AND PLASMA.

CONTROL SERIES

Antigen	Specific Amboceptor	Guinea-Pig Serum*	Hemolysin	Cells	Result
3 drops	2	3	2 units (4C)	25 per cent	o
3 drops	3	4	2 "	25 " "	o
3 drops	4	5	2 "	25 " "	o
4 drops	5	6	2 "	25 " "	o

TEST SERIES

Antigen	Specific Amboceptor	Plasma†	Hemolysin	Cells	Result
4 drops	4	1	2 units	25 per cent	6‡
4 drops	4	2	2 "	25 " "	6
4 drops	4	3	2 "	25 " "	4
4 drops	4	4	2 "	25 " "	6
4 drops	4	5	2 "	25 " "	6
4 drops	4	6	2 "	25 " "	6

* Diluted 1 to 2.

† Undiluted.

‡ Complete hemolysis at the end of 15 minutes.

This experiment and other similar ones appear to be capable of interpretation in but one way, namely, that complement in a form available for binding in the presence of antigen and specific amboceptor does not exist in plasma as procured from guinea-pigs' blood; it appears furthermore, that by the addition of a suspension of washed erythrocytes, some body is added which renders the plasma active at least in so far as its hemolytic power is concerned. It is but just to note that similar results are not obtained in all instances; I believe, however, that, in dealing with a material which is as difficult to procure as guinea-pig plasma, a few positive results are of more importance than a number of negative findings.

In all my experiments in which plasma has been added to a mixture of normal salt solution and either red blood cells or bacterial antigen, fibrin formation has consistently occurred. This fact is mentioned in view of the apparent importance attached to this phenomena by Addis.[1]

[1] *Jour. Infect. Dis.*, 1912, 10, p. 208.

DISCUSSION.

It is my belief that complement as we are accustomed to consider it, in ordinary *in vitro* experiments, does not exist as such in the circulating plasma, but that a body which may be termed complementogen is present in practically constant amounts. This complementogen is rendered active only as a result of the liberation of some substance, probably similar to thrombo-kinase, which is produced chiefly by white blood cells.

These views are based on the facts observed and recorded above; namely, that the amount of complement demonstrable in serum varies with the length of time and the temperature at which the blood is kept, thus suggesting that it is a development following removal from the body; the more so since a temperature of 37.2°, or slightly higher, is more uniformly followed by a rapid production of the body than if a lower temperature be employed. There is no reason for believing, according to this hypothesis, that complement may not be produced as occasion may arise within the body, owing to the interaction *in vivo* of the two substances just mentioned. In order to harmonize Addis' results in experimentation on cock's serum I suggest the possibility that inasmuch as fowl's blood submits to relatively greater insult without coagulum formation, the complementogenic substance is acted upon with free complement formation during the time consumed in preparing the fluid. In other words, it may be that the complement-inducing substance is liberated in a shorter period of time than that necessary for the formation of a sufficient quantity of thrombo-kinase to bring about clotting.

SUMMARY AND CONCLUSIONS.

The maximum complement content of a given specimen of serum depends upon the temperature at which the blood is kept following removal from the body. The more nearly this temperature approaches that of the body the greater is the amount of complement developed and the more rapidly does it appear.

The maximum complement content of healthy animals of the same species is remarkably constant in amount.

Following the maximum development of the complementary

body there occurs some change, the nature of which has not been discussed in this paper, resulting in the reduction of the amount of complement available for hemolytic experiments. This depreciation in activating power of the serum occurs more rapidly at higher than at lower temperatures, being practically absent at temperatures of 0° C.

In the ordinary hemolytic experiments employed in the estimation of complement content of plasma, a relatively small amount of complement is demonstrated. The author has not, however, been able to prove the absence of this body in plasma by the employment of this method.

By means of the Bordet-Gengou complement binding reaction it is possible to prove that no complement susceptible to fixation in the presence of an excess of specific amboceptor and bacterial antigen is present in plasma.

From a consideration of the above facts the author concludes that complement is not a normal constituent of animal plasma but that there is present a pre-complement or "complementogen" which, when acted on by or in combination with some substance produced under stimulation of the blood, by certain body cells including the leukocytes, forms the finished complementary product.

From a practical point of view these experiments demonstrate that the serum of guinea-pigs, unless they be the subjects of acute infections, may be depended on to contain a constant amount of complementary body available for serum reactions, granted that the blood from which it is procured is preserved in a definitely standardized manner especially in so far as this refers to temperature and time. Conversely, blood kept in haphazard manner cannot be depended upon for employment in complement-binding reactions.

I have found that if, on removal from the body, the blood be placed at 37° C. for a period of from one-half to one hour, and subsequently placed in the ice-chest at a temperature of about 2° C. it may be employed with but little likelihood of experimental error arising therefrom during a period of from 24 to 48 hours.

ON THE NATURE OF THE TOXIC SUBSTANCE FROM PNEUMOCOCCI.*

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The close relation between disintegration of bacteria in general and the production of soluble toxic substances, as I have shown,[1] might be interpreted as meaning that preformed endotoxin is liberated. The fact that the toxic substances obtained by autolysis of the various species are identical in their action speaks against this idea. I have shown that the toxic substances are absent at first, that they appear at a certain stage of autolysis, and then disappear; hence it would seem that they are produced during the disintegration of the bacteria. In the salt solution suspensions I have shown by means of the polariscope and formol titration that this appearance and disappearance of toxic substances are associated with proteolysis. That it is not a question of solution of toxic material which it may be assumed is contained as such within the pneumococcus is shown further in the following experiments, in which proteolysis was prevented for a time and in which the pneumococci were broken up mechanically by grinding them with sterile, washed cold sand and cold salt solution in a mortar. Care was exercised in this experiment to select a strain of pneumococcus not too virulent, because in those of exceptionally high virulence there are already present in 24-hour cultures many pneumococci undergoing autolysis. After grinding to the point when only an occasional pneumococcus could be found, and suspending the triturated mass in cold NaCl solution for two hours, the suspension was centrifugated clear. Seven cubic centimeters were then injected at once into the jugular veins of two normal guinea-pigs, weighing 200 gms. No immediate symptoms developed; in three hours the animals seemed ill, and both were dead the next morning from pneumococcemia. Similar results were obtained in others injected after the suspension had been kept

* Received for publication July 13, 1912.

[1] *Jour. Infect. Dis.*, 1912, 10, p. 113.

at 37° C. for one, three, and six hours. The two at the end of six hours showed mild symptoms in three minutes after the injection. Those injected at the end of 18 hours died from typical symptoms in three minutes, while those injected at the end of 48 hours showed no symptoms and remained permanently well. Formol titration showed a definite increase in amino-nitrogen. A second experiment gave the same results.

In the light of these experiments and the other facts, the conclusion seems warranted that the toxic material which produces the acute death from bronchial spasm is produced at least in large part during retrogressive changes and does not exist preformed within the pneumococci.

The delayed toxic effect of pneumococcus extracts is greatest when there is present the largest amount of the substance which provokes bronchial and other spasms. Animals which survive the immediate symptoms often die in 18 to 24 hours with extensive hemorrhages. It seems therefore that it concerns more than one toxic substance. Without further proof, however, we could not be sure that the late death might not be due in part at least to the early damage of the cells produced by the substance which causes acute death from bronchial spasms. Hemorrhages occur in both instances, although those following acute death from bronchial spasm are relatively slight and occur chiefly in the lungs and diaphragm. Those observed in other regions are what one might expect from acute asphyxia from any cause. I have tried by various methods to remove the toxic substance which kills the animal by producing bronchial spasm so that the effect of larger doses of the material which seems to be responsible for the late death could be studied. That the substance which kills by producing bronchial spasm is capable of rapidly attaching itself to the cells is indicated by its exceedingly rapid action. Hence various adsorbing substances have been tried and it has been found that animal charcoal, particularly blood charcoal, removes this substance rapidly from toxic extracts. Thus 0.1 gm. per 10 c.c. of extracts is sufficient to remove all toxic material in one hour. An extract which kills in two to three minutes from typical symptoms causes no immediate symptoms after such treatment, the animal remaining permanently

well. If, however, twice that dose of charcoal-treated autolysate is injected, the animal is apt to die in 18 to 24 hours but with no immediate symptoms referable to the respiratory tract. The following observation also has been made in connection with the effect of animal charcoal on pneumococcus autolysates: When the toxicity is high the removal of the toxic substance is associated with a reduction in the formol titration figure, whereas after treatment of autolysates which have been allowed to become nontoxic, reduction in the titration figure is less pronounced. The explanation of this fact is not clear but it might be suggested that the toxic substance has a great affinity for the charcoal and that it carries with it amino-acids and in that way lowers the titration figure. From observations made on amino-acids we know that the respiratory symptoms are not due to these substances. Similar results have been obtained with animal charcoal in case of toxic mixtures prepared with serum and in autolysates reactivated by the addition of serum.

Barium sulfate does not remove the toxic substance. Living and heat-killed, virulent pneumococci do not remove the toxic substance from the autolysates but render them more toxic. Living nonvirulent pneumococci and autolyzed virulent pneumococci on the other hand diminish the toxicity slightly.

In my previous papers it is shown that ether, while not essential, makes it easier to obtain the highly toxic effect of bacterial autolysates. It was suggested that ether killed the bacteria and thus intensified the reaction. The possibility of the substance being soluble in ether which thus serves to extract it from the disintegrating bacterial cell also suggests itself. This point has been studied, and it has been found that the substance which kills by bronchial spasm can be separated completely from toxic autolysates by ether extraction. The results in Table 1 are typically illustrative. It is seen that as the toxic autolysate or extract loses its acute toxic effect by the extraction, and that the ether soluble residue, suspended in an equivalent amount of NaCl solution, becomes toxic in direct proportion. The amount of amino-nitrogen lost in the broth is equal to that found in the ether extract. This fact suggests strongly that the toxic substance contains nitrogen, and because

it is available in the formol titration, it is probably an amin. In order to remove all this toxic substance it is necessary to use large quantities of ether (three to six times the amount of extract) and to shake thoroughly for 10 to 20 minutes in each extraction, the substance being soluble in ether with some difficulty. The fact that ether fails to remove this toxic substance when partially autolyzed pneumococci have not been removed from the extract shows that they have a certain affinity for the toxic substance. This undoubtedly explains why it is more difficult to obtain free in the NaCl solution a sufficient amount of toxic material in the absence of ether because digestion within the pneumococci may go beyond the toxic stage before the product is free in the NaCl solution.

TABLE 1.

EXTRACTION OF ACUTELY TOXIC SUBSTANCE WITH ETHER.

Mixtures (Extract 284 was prepared from two strains of highly virulent pneumococci [617 and 618], grown in meat broth for 24 hours, washed once, suspended in NaCl solution in the usual way, and placed at 37° C. for 48 hours.)	Symptoms on jugular injection in (duplicate) guinea-pigs
8 c.c. Extract 284 centrifugated clear	Death in 4 minutes
8 c.c. Extract 284 with pneumococci not removed	Death in 3½ minutes
8 c.c. Extract 284 clear and after 3 extractions in 3 times the amount of ether	No immediate symptoms, death with hemorrhages in 8 hours
8 c.c. Extract 284, pneumococci not removed, treated with ether as above	Death in 4 minutes
Ether soluble substance from 8 c.c. of clear extract suspended in 8 c.c. of NaCl solution	Death in 2 minutes from typical symptoms, blood very dark
Ether soluble substance from 8 c.c. of extract but with pneumococci not removed suspended in 8 c.c. NaCl solution	Slight symptoms, severe scratching, irritability, etc.

As soon as it was found that the acutely toxic substance was soluble in ether (Mallenkrodt's anesthetic ether was used) an attempt was made to extract it from highly virulent and unautolyzed pneumococci when suspended in NaCl solution, and when dried, but without success. When toxic pneumococcus autolysates are evaporated to dryness at room temperature, the residue ground up in a mortar with sand, and then extracted with ether, only a small amount of highly toxic substance is found in the ether residue. It is impossible to say whether the toxic substance is more soluble in

the "watery" ether or destroyed during evaporation. It has also been extracted with ether from toxic autolysates of diphtheria bacilli as well as from extracts of pneumococci made toxic with immune serum and normal serum, with normal serum alone, and with leukocytes, and also from the clear broth culture filtrates of pneumococci. Moreover, by shaking ordinary peptone broth with ether and to a lesser degree dry powdered Witte peptone, an acutely toxic substance which produces similar symptoms is obtained.

Quantitative studies on the action of clear toxic autolysates and the ether soluble substance suspended in equivalent amounts of NaCl solution show that the latter are even more toxic than the former. The symptoms begin earlier, the respiratory efforts are not so violent, dose for dose, because the animal is overwhelmed too rapidly. If approximately three-fourths of the ether soluble substance of a measured toxic dose of the extract is injected, the spasms may be as pronounced as when the extract itself is injected. When the extracts fail to produce symptoms, then extraction with ether fails to remove any toxic material. Animal charcoal removes the toxic substance from NaCl solution suspensions of the ether soluble residue. Animal charcoal, which has taken up the toxic substance, gives up only a part of this substance when shaken with ether just as ether fails to remove in appreciable amounts the toxic substance from pneumococcus suspensions.

In Table 2 is shown the effect of blood charcoal and ether on the formol titration and toxicity of pneumococcus broth culture filtrates. There was used 0.2 gm. of the charcoal per 10 c.c. of the clear culture fluid. It was allowed to act for one hour at 37° C. The extractions with ether were repeated three times in the proportion of two parts of ether to one part of broth. There was observed here as in the autolysates a reduction in formol titration and toxicity.

When clear toxic pneumococcus autolysates are heated to 60° C. from five to 20 minutes the toxicity for guinea-pigs disappears. When, however, suspensions of pneumococci have become highly toxic after having been kept at 37° C. for 24 to 48 hours and there are present a fair number of well preserved pneumococci the toxicity does not disappear nearly as easily.

Study of this peculiar result shows that if the heat is brought

up to 60° C. very gradually the toxicity might entirely disappear in from one to three hours, but if the suspension was plunged into boiling water the toxicity would frequently remain even after boiling for 10 minutes. This heated suspension would now remain toxic for a long time when kept on ice.

TABLE 2.

THE EFFECT OF BLOOD CHARCOAL AND ETHER ON THE TOXICITY AND FORMOL TITRATION OF PNEUMOCOCCUS BROTH CULTURE FILTRATES.

Mixtures	Formol Titration	Symptoms in (duplicate) guinea-pigs following injection of 4.5 c.c. intravenously
Meat broth culture pneumococcus 622[4]	2.1 4.2	Nearly dead in 3 minutes, dead in 20 minutes
Same after treatment with animal charcoal	1.6 3.3	Slight symptoms in 10 minutes
Same after ether extraction	2.1 3.9	Definite symptoms
Meat broth control*	.65 2.2	Definite symptoms

*The ether extract from 16 c.c. of the broth culture fluid produced marked symptoms, while that from the broth treated in exactly the same way gave no definite symptoms.

An analogous observation has been made with pneumococcus broth culture fluids. When the pneumococci are removed heating reduces the toxicity markedly, whereas, when the organisms are allowed to remain, heating reduces their toxicity only slightly or not at all. Similar results have been obtained with extracts of pneumonic lungs. It seems, therefore, that the rapid disappearance of the toxic property of the clear extracts is due to ferment action. The toxic substance is converted rapidly into nontoxic material, but where there is present a supply of material which has not yet reached the toxic stage, new toxic material is made until the ferment itself is destroyed and heating no longer destroys the toxic substance. It seems, therefore, that the toxic substance is really thermostable and not thermolabile as one would be led to believe from the effect of heat on the clear extracts.

The effect of acids and alkalies on the toxic substance has also been studied. The reaction of the autolysate is practically neutral. If clear toxic pneumococcus autolysates are made 2 per cent acid with hydrochloric acid and this at once neutralized with sodium

carbonate or sodium hydrate, it is found that the substance which provokes bronchial spasm has entirely disappeared. Lower concentrations of acid require a longer time until all the toxic substance disappears. If other portions are alkalinized to the same degree and neutralized with hydrochloric acid, the toxic action is reduced to a lesser degree. Similar but less striking results have been obtained with pneumococcus broth culture filtrates. It would therefore seem that the toxic substance is an amino-base and might very well be B. imidoazolylethylamine which Dale and Laidlow[1] have isolated from Witte peptone and which they believe to be the substance which provokes bronchial spasm in guinea-pigs.

By means of the ether I am thus able to separate, in more or less pure form, the toxic substance from the other protein constituents of the pneumococcus autolysates.

The question whether intoxication and immunization during infections are due to the same cause, or whether substances are formed which not only intoxicate without at the same time calling forth antibody formation, but even interfere with antibody production, is still unsettled. I have shown that intravenous injections of extracts of pneumococci before, during, and especially after the toxic stage protect guinea-pigs against subsequent injections of toxic autolysates. This is specific for pneumococci. It has now been found that nonfatal doses of the toxic, ether soluble material does not protect against toxic autolysate or against toxic ether soluble material. The autolysates on the other hand, both before and after ether extraction, protect against the toxic ether soluble material and toxic autolysates.

CONCLUSIONS.

The toxic substance obtainable from pneumococci has been found to be soluble in ether. It is formed chiefly during retrogressive changes in pneumococci. Heating the clear toxic autolysate to 60° C for 20 minutes destroys the toxicity, while toxic pneumococcus suspensions remain toxic even after boiling Hydrochloric acid in weak solution destroys the toxicity of pneumococcus autolysates. The toxic substance is adsorbed by blood charcoal

[1] *Jour. of Physiol.*, 1910, 41, p. 318.

from which it can again be obtained by shaking with ether. Autolyzed virulent pneumococci and nonvirulent pneumococci diminish the toxicity slightly while unautolyzed virulent pneumococci increase it. The toxic substance is probably a base which contains amino groups of nitrogen.

Indications have been obtained showing that during pneumococcus infections toxic substances are produced which do not call forth any immunizing response.

EXPERIMENTAL "RHEUMATIC MYOCARDITIS."*

EXPERIMENTAL STREPTOCOCCAL FOCAL LESIONS IN THE HEART MUSCLE OF RABBITS SIMILAR TO LESIONS IN THE HUMAN HEART IN RHEUMATISM.

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Good accounts of the structure of the nodular lesions in the heart muscle in rheumatic myocarditis are found in the articles by Aschoff,[1] Geipel,[2] Coombs,[3] Gallavardin,[4] and others. In most of the accounts of these alterations credit is given to Romberg[5] for first reporting their occurrence. Since the nodules are on the border-line of visibility, they doubtless have been seen by many observers, and careful search of the literature might disclose that others besides Romberg have described such changes and like Romberg failed to attach much significance to them.

One such observation about the same time as Romberg's is that by Bret.[6] In describing the changes in the heart muscle of a girl of seventeen who died from acute rheumatism with pericarditis, he mentions discrete localized changes deep in the muscle unconnected with the pericarditis, made up of embryonal cells and located about the septa.[7]

By a number of writers the foci are referred to as the "Aschoff-Geipel nodules." Certainly the credit for suggesting the specific

* Received for publication July 30, 1912.

1 *Verhandl. deut. path. Gesellsch.*, 1904, 8, p. 46; Aschoff and Tawara, *Die heutige Lehre von den pathologisch-anatomischen Grundlagen der Herzschwäche*, Jena, 1906; *Brit. Med. Jour.*, 1906, 2, p. 1103.

2 *Deutsche Arch. f. klin Med.*, 1905, 85, p. 75; *München. med. Wchnschr.*, 1909, 56, p. 2469.

3 *Brit. Med. Jour.*, 1907, 2, p. 1513; *St. Mary's Hosp. Gaz.*, 1910, 16, p. 121; *Jour. Path. and Bact.*, 1910–11, 15, p. 489.

4 *Lyon med.*, 1908, 110, p. 753, and 1911, 117, p. 149.

5 *Deut. Arch. f. klin. Med.*, 1894, 53, p. 141.

6 *La Province Méd.*, 1894, 8, p. 437.

7 The more significant of his observations of the changes are stated as follows: "Indépendament de ces foyers de prolifération embryonnaire périvasculaire, il existe, dans la profondeur du muscle cardiaque, à une distance notable du péricarde inflammé, d'autres foyers que paraissent absolument indépendants de toute connexion vasculaire ou conjonctive. Ce sont pour la plupart des amas arrondis ou des traînées culaires, dans l'intervalle de ces derniers. Ils offrent une constitution uniforme qui est à peu près la suivante: la partie centrale de ces foyers inflammatoires est formée de blocs opaques caséifiés, fixant très fortement la couleur; ils semblent résulter, selon nous, de la dégénérescence des fibres musculaires cardiaques. A leur pourtour se dispose une multitude d' éléments embryonnaires."

relationship of the nodules to the infectious agent of acute articular rheumatism may be properly given to Aschoff and Geipel. In 1909 Geipel states that the total number of cases in which nodules had been found, and associated with rheumatism by the writers, was 24. He fails to include in this enumeration the four cases of Saigo referred to by him in his first article.

Geipel, and following him a number of writers, have found it difficult to account for their presence in the heart muscle of a boy of eleven who died of chronic nephritis, obliteration of the pericardium, and verrucose mitral endocarditis, on account of the absence of joint involvement clinically. With this exception, the legitimacy of which as an exception may very well be questioned, there are but few comments in the literature pertaining to these lesions in the human myocardium opposing their specific limitation to rheumatism.

The most recent advocate of their etiology in the rheumatic infection is Fraenkel,[1] who found them in 17 of 20 cases with a history of rheumatism. He concludes that although not present perhaps in the hearts of all who have had acute articular rheumatism, their presence indicates that infection with the agent of this disease has at some time taken place.

In the course of examinations of animal reactions to streptococci, to which reference has been made by D. J. Davis,[2] opportunity has been afforded to examine the heart muscle of rabbits injected with various strains of streptococci. All of the streptococci were of the type described by Davis and Rosenow[3] in the recent milk epidemic of sore throat in Chicago, with exception of two, a streptococcus viridans and a hemolytic streptococcus.

Changes in a rabbit killed two hours after a single intravenous injection of the 24-hour growth at 37° C. on two blood agar slants of a streptococcus (236) isolated by Dr. Davis from the serofibrinous peritoneal exudate found at necropsy, and producing arthritis in rabbits. The peritonitis was a sequence of sore throat during the milk epidemic.[4]—Sections of the heart muscle contain small groups of cells quite uniformly distributed. Any low-power field of these sections contains from three to six or eight of these lesions. They occupy the space between one or more muscle fibers with their long axis parallel with the fibers, and contain from 10 or 15 to 100 cells. The most conspicuous and usually the

[1] *Beitr. sur path. Anat. u. z. allg. Path.*, 1912, 52, p. 597.

[2] *Jour. Am. Med. Assoc.*, 1912, 58, p. 1283. [3] *Ibid.*, p. 773.

[4] J. A. Capps and J. L. Miller, *ibid.*, p. 1848.

larger number of cells are large with large oval or round, sometimes irregular, nuclei which stain unevenly and rather lightly and may be centrally or a little eccentrically placed. The cytoplasm stains faintly and forms a rim about the nucleus about one-third the diameter of the nucleus. The size of the cells varies considerably and it is not difficult to find cells of this type containing two, sometimes six or eight streptococci. In addition to these large cells and almost as numerous, are smaller cells with more deeply stained nuclei and a small amount of cytoplasm. They are irregularly disseminated as are also a few polymorphonuclear leukocytes.

Changes in a rabbit killed 10 hours after a single intravenous injection with the same streptococcus (236) using the growth from four blood agar slants after 24 hours at 37° C.—There are large collections of bacteria in the minute blood vessels. No necrosis is observed about these collections nor is there any cellular infiltration about them.

Changes in a rabbit killed 24 hours after a single injection of the growth on two blood agar slants of the same streptococcus (236).—The lesions which are most conspicuous and numerous are about 0.25 mm. in diameter, rounded or oblong in shape according to the direction of the section in relation to the muscle fibers. They contain solid masses of bacteria separating or replacing necrotic muscle fibers. There is a tendency in some of the lesions for the masses of bacteria to appear at the periphery, leaving the center fairly clear or containing scattered chains of cocci and necrotic muscle fibers, in others the bacteria are more evenly distributed, and in still others the bacterial masses are very definitely outlined as though occupying the lumen of a vessel. There is no infiltration of cells in the vicinity of these regions and the muscle fibers immediately surrounding them are necrotic. Other minute focal lesions consisting of collections of mononuclear cells occur but are not numerous, one or two being found in some sections, in others, none. The cells in these lesions are large, lightly stained, irregularly shaped, with large round or oval nuclei, and smaller cells with more deeply stained nuclei. The two varieties occur in about equal numbers. Some of the large cells contain bacteria. A few scattered chains of cocci are sometimes present in the periphery of the lesions.

Changes in a rabbit dying spontaneously two days after a single intravenous injection of the 24-hour growth on one blood agar slant of a streptococcus (214) isolated by Dr. Davis from suppurating lymph glands secondary to a sore throat.—There are many necrotic regions containing bacteria. These lesions are round, oval, or spindle shaped according to the direction of the section, and some of the largest lesions are 1.5–0.6 mm. in diameter. The organisms are frequently arranged in clumps, distributed rather uniformly through the central portion, in others they form a ring near the periphery, and in a few, one large, dense mass. Necrosis is a marked feature of these lesions, and muscle fibers in the center of some stain faintly and have partially disappeared. The necrosis often extends for a considerable distance beyond that portion of the lesion occupied by bacteria and may equal the width of two or three fibers. On the other hand, a few lesions possess but very slight necrosis about the bacteria and there is a general infiltration with polymorphonuclear cells. There is considerable nuclear fragmentation in these cells, and about the borders of these regions there are many eosinophiles. A slight modification of the alterations described consists of small collections of large and small mononuclear cells about blood vessels in about equal numbers, together with some polymorphonuclear leukocytes and eosinophiles. Bacteria are found in some of the large mononuclear cells in these lesions but they do not occur in clumps.

Changes in two rabbits dying spontaneously three days after single intravenous injections of the 24-hour growth on blood agar slants of (1) a streptococcus (219) isolated by Dr. Davis from the pus obtained during life from a brain abscess following sore throat, and (2) streptococcus (233) obtained from the spinal fluid during life in a case of meningitis following sore throat.—Sections from the heart muscle of these rabbits present lesions which are in many respects similar, and, with a few exceptions, one description will answer for both. The lesions containing bacteria without any cellular infiltration average about 3–5 cm. in section, 1–0.5 cm. in diameter. They are larger and associated with more necrosis in the muscle of Rabbit 1. In a few the bacteria are arranged in dense rounded masses but in one heart muscle they occupy the intermuscular spaces and tend to form a meshwork or one or more long narrow bands sometimes definitely outlined as though filling the lumen of a vessel. Others are frequently located near blood vessels and arranged as a wall on one or both sides of the blood vessels. In addition to such changes there are also small collections (Fig. 1) of mononuclear cells and in about the same frequency as the patches of necrosis with bacteria. These collections of cells vary considerably in size, some containing 15–20, others, 100 or more cells. By far the majority are large mononuclear cells, small mononuclears comprising about one-third. Polymorphonuclear leukocytes are very rare.

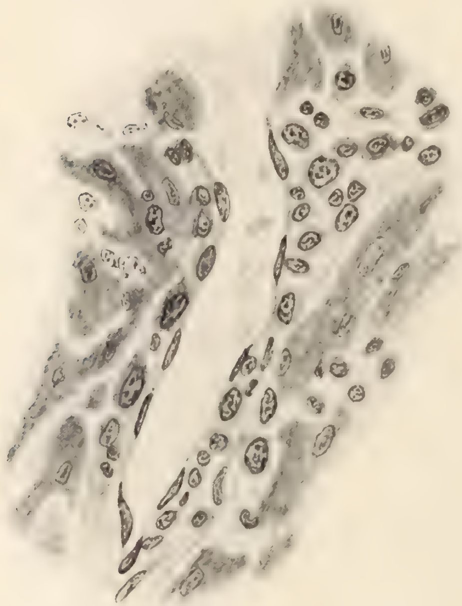

FIG. 1.—Perivascular collection of large mononuclear cells in the heart of a rabbit dying three days after inoculation of streptococcus (219) from a brain abscess following sore throat.

In a third variety of focal inflammation, bacteria are distributed throughout and there is a general infiltration with polymorphonuclear leukocytes and mononuclear cells with a considerable amount of nuclear fragmentation.

In some of the sections of a series there were typical abscesses with central masses of bacteria radially arranged at the margin with zones of polymorphonuclear leukocytes about them; still more externally, narrow zones of necrotic muscle fibers, the whole surrounded by muscle fibers pushed to either side. In tissue surrounding these abscesses polymorphonuclear leukocytes are quite abundant but scatteringly arranged.

Changes in a rabbit dying spontaneously five days after a single intravenous injection of the 24-hour growth on a single blood agar slant of a streptococcus (35) sent to Dr. Davis

by Dr. Fabyan of Boston, and isolated from erysipelas complicating sore throat in the Boston milk epidemic.—There are small lesions (17 in sections 2–0.3 cm. in diameter), most of them near the outer surface of the heart. In many of them bacteria are found filling the lumen of vessels. In some only bacteria are present, in others there is a rather uniform admixture of disintegrating cells. In places about these lesions are collections of large endothelial cells with a few polymorphonuclear leukocytes, containing considerable nuclear fragmentation.

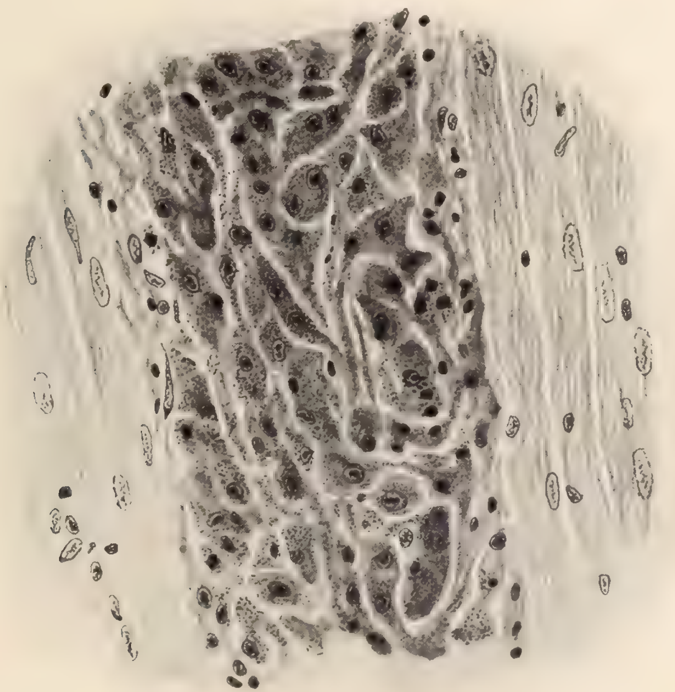

FIG. 2.—Part of a lesion consisting of large mononuclear cells closely crowded together and filled with organisms. From the heart of a rabbit dying 13 days after injection of streptococcus (236) from peritonitis secondary to sore throat.

Other lesions which cannot be so definitely connected with blood or lymph vessels consist of bacteria and disintegrating muscle fibers and cells with marked nuclear fragmentation in and around them. About these there is a very little necrosis of the muscle fibers, and polymorphonuclear leukocytes are quite abundant between the fibers.

Changes in a rabbit dying spontaneously seven days after a single intravenous injection of a 24-hour growth of a streptococcus (218) isolated by Dr. Davis from the serofibrinous exudate of pleurisy following sore throat.—There are but few lesions in these sections.

Two or three collections of from 15–25 cells are found in some sections, in others one somewhat larger lesion is found. The small collections contain chiefly large mononuclear cells rather lightly stained which have large, oval, unevenly stained nuclei. Many of these cells contain organisms. With these cells are smaller ones which have more deeply stained round nuclei. There is no necrosis about these regions. The

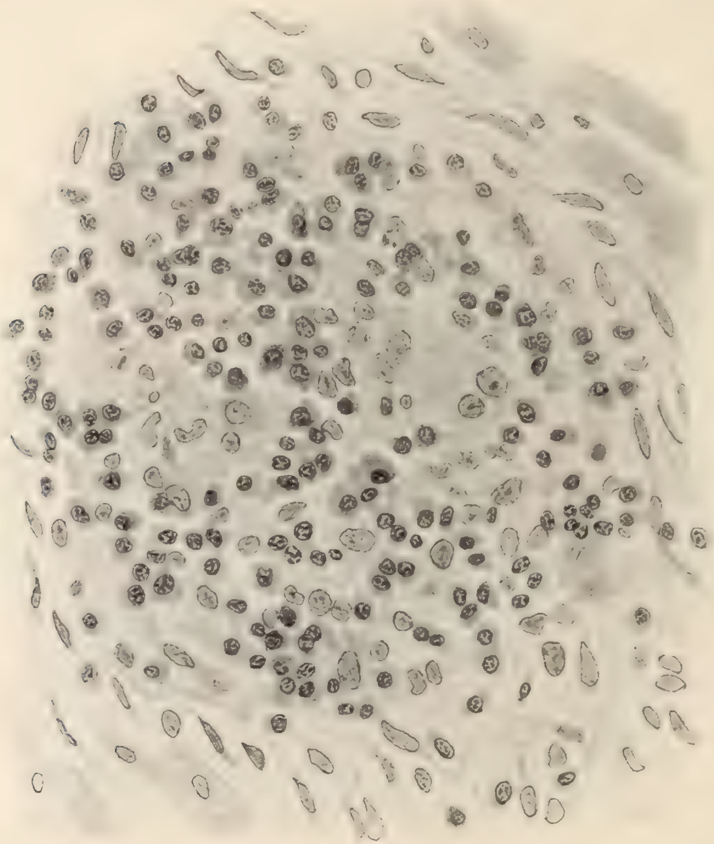

FIG. 3.—Mononuclear cells in a focal lesion from the heart of a rabbit dying two months after the first injection of streptococcus viridans (169).

largest lesion practically fills the field (Obj. 1/12, Oc. 1), and consists of a few remnants of necrotic muscle fibers, many large mononuclear cells with lightly stained granular cytoplasm, and a few smaller cells with more deeply stained nuclei, and a considerable number of new connective tissue cells.

Changes in a rabbit killed 13 days after a single intravenous injection of a 24-hour growth of streptococcus (236) on a single blood agar slant.—Three lesions are found in

sections taken from two different places. The largest is about 1.5–0.5 mm. in diameter with its long axis in the direction of the muscle fibers. The other lesions are less than half as large and of the same character. They are composed of large mononuclear cells of variable size and indefinite outline closely crowded together. Nearly all these cells contain organisms so numerous in some cells that they cannot be counted with accuracy; in others they number from 10–50. There are no polymorphonuclear leukocytes within or about these lesions (Fig. 2).

Changes in a rabbit dying during intracardial injection of a 24-hour growth on two blood agar slants of a streptococcus viridans (169) obtained by Dr. Davis from the heart

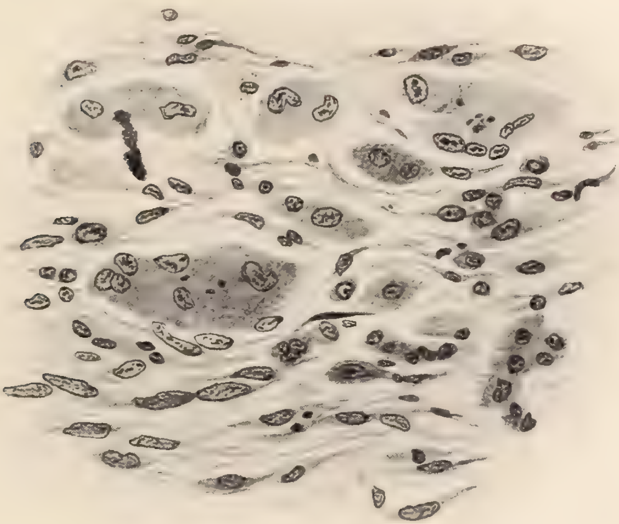

FIG. 4.—Nodule with multinucleated cells in the heart of a rabbit dying two months after the first injection of streptococcus viridans (169).

valves of a man who long had suffered with endocarditis. The same streptococcus was found during life in the tonsils. Numerous tonsillar abscesses were present. This streptococcus produced endocarditis but not arthritis in rabbits. Three other intravenous injections had been made in this rabbit over a period of two months at intervals of 14, 21, and 14 days.—The largest number of lesions contain degenerating muscle fibers which stain more deeply than the normal fibers and in some of the lesions they stain very intensely. Surrounding many of the fibers or grouped at one side are several nuclei which give them the appearance of giant cells. Large mononuclear cells are present in all these regions, sometimes arranged in groups but generally scattered in not very large numbers quite evenly throughout the lesion. Large multinucleated giant cells are also present in many of the lesions (Figs. 3 and 4). There is an increase in connective tissue in and about these regions. There is also a considerable number of lesions which contain no degenerating muscle fibers and are composed almost entirely

of mononuclears (Figs. 3 and 4). In some, the cells occupying the center of the lesion are very large with round or oval lightly stained nuclei and a large amount of granular cytoplasm in which bacteria are often seen. The bacteria are not numerous and in marked contrast with the great numbers found within similar cells in Rabbit 236 dying 13 days after inoculation. The outlines of these cells are not sharply defined. About the center are many smaller, more deeply stained cells, which are irregularly shaped and definitely outlined, and have round, rather deeply stained and eccentrically placed nuclei. The cytoplasm is fairly abundant, the width of the rim varying from a fourth to the entire diameter of the nucleus. In a few of these lesions are also a considerable number of polymorphonuclear leukocytes many of which are eosinophiles. A few of these lesions are not sharply marked off from the surrounding tissue, but there is a diffuse infiltration of cells among the muscle fibers. One lesion, which differs from the rest, consists of a vessel wall which is occupied by a pink-stained, homogeneous material surrounded by polymorphonuclear leukocytes, many of which are eosinophiles, together with a few large mononuclear cells. The vessel wall is infiltrated with leukocytes and mononuclear cells and a short distance away are small collections of cells which possess many of the features of plasma cells.

Changes in a rabbit killed 24 hours after intravenous injection of the 24-hour growth on blood agar slants of the streptococcus viridans already referred to (169). Other injections had been given over a period of three months at intervals of 4, 8, 7, 2, 14, 16, and 12 days each. The first two were of the 24-hour growth on one blood agar slant of a streptococcus mucosus (28) isolated by Dr. Davis from the middle ear. Each of the remaining injections were of the 24-hour growth on five blood agar slants of the streptococcus viridans previously mentioned.—Sections were made from three different places and lesions found in all. Some are small, not numerous, of the cellular type, containing 15 to 50 cells, generally mononuclears. Others, which are several times larger, are made up of polymorphonuclear leukocytes and mononuclear cells without necrosis. In some of the larger ones the polymorphonuclear leukocytes are more numerous, the center is occupied by leukocytes surrounded by a rather uniform mixture of leukocytes and mononuclear cells with a considerable number of eosinophiles scattered throughout. Both the smaller and the larger lesions are not sharply demarcated, and the muscle fibers appear quite unchanged.

In the relative absence of necrosis and of polymorphonuclear leukocytes no less than in the abundance of, and apparently large rôle played by, the large, so-called "endothelial" phagocytic leukocytes, the focal lesions produced in the heart muscles of these rabbits are peculiar. These characteristics are maintained throughout all the varying phases of their development, and they are the features which have been emphasized especially by Coombs but also by others, as distinctive of the focal lesions in the human heart in rheumatism. All of the streptococci, except the viridans (169), produced arthritis in rabbits. The differences in the changes are not marked when allowance is made for the varying periods at which the examinations were made. The oldest lesions, those in

rabbits killed two and three months after injections of streptococcus viridans, were the only ones in which giant cells were found, which different writers have likened to the multinuclear cells in certain sarcomas and in the pseudoleukemic process. The alterations vary so little in their general nature as studied in the varying stages of their development that it seems reasonable to expect that other streptococci than the viridans may also produce focal changes with these peculiar giant cells.

Wächter[1] mentions having produced changes in the rabbit's heart resembling those of human rheumatism with a streptococcus viridans isolated from the heart's blood at the postmortem examination of a man aged 29, who died of an acute articular rheumatism. The chief result of subsequent experiments reported by Bracht and Wächter[2] was the production of changes in rabbits' hearts which were different from those caused by the ordinary streptococcus pyogenes (hemolyticus) but not identical with those in the human myocardium.[3] In the endeavor to produce myocarditis in rabbits, Bracht and Wächter used streptococci recovered from the blood of the heart chambers of two individuals dying from rheumatism. Of 10 rabbits seven were found to have a myocarditis. The authors describe the changes in three of them. One of their illustrations shows lesions quite similar to those I found were produced by the milk-epidemic streptococci as well as those caused by the viridans. Bracht and Wächter used 24-hour broth cultures and several intravenous injections of from 4 to 5 cm. The changes reported were observed in rabbits killed on the 9th, 10th, and 16th days. In five of the seven rabbits an endocarditis was present. In a number of the hearts I examined, endocardial lesions were also present. The examinations I have made of changes produced in the rabbit heart by an ordinary hemolyzing streptococcus pyogenes are too few to compare with those described in the foregoing protocols.

The experimental production in rabbits of these minute, disseminated regions of infection, their close morphological correspondence to the focal lesions in the human heart in acute articular rheumatism, and the fortunately exceptional characteristics of the

[1] *München. med. Wchnschr.*, 1908, 55, p. 1101.
[2] *Deutsche Arch. f. klin. Med.*, 1909, 96, p. 493.
[3] L. Aschoff, *Pathologische Anatomie*, Jena, 2d ed., 2, p. 33.

changes, which make comparison so easy, obviously lead to the suspicion that they all have a similar etiology. As is well known, the ordinary streptococcus pyogenes produces in man, as well as in rabbits and other animals, minute abscesses with a marked infiltration of polymorphonuclear leukocytes, changes quite different from those forming the subject of this report. It may be well also to recall that involvement of the joints took place in many of the patients with epidemic sore throat, from whom the streptococci used to produce these lesions were obtained.

A STUDY OF THE DIPHTHERIA GROUP OF ORGANISMS BY THE BIOMETRICAL METHOD.*

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The relationship of the diphtheria bacillus to the so-called "diphtheroids" and the "pseudo-diphtheria" or Hofmann's bacillus, has been frequently discussed. Much work has been done on the changes in morphology of the diphtheria bacillus under various conditions; also on the comparative cultural characteristics of the diphtheria bacillus, Hofmann's bacillus, and the diphtheroids, and on the variations in virulence of the true Klebs-Loeffler organism. Attempts have been made to transform Hofmann's bacillus into a typical diphtheria bacillus, and vice versa. A small amount of work has been done on the serum reactions in the group.

The view which has gradually come to prevail, forced largely by public health work, is that, whatever the systematic biological relationships of the organisms may be (and this is left an open question), practically at the present time the "diphtheroids" and "pseudos" should not be classified with the true Klebs-Loeffler bacillus.

It was thought that the biometrical or statistical method, which has been applied with much success to the coccaceae by the Winslows,[1] might prove of equal value in the diphtheria group, to determine whether or not subdivisions exist, and also what is the relationship of the "diphtheroids" and "pseudos" to the true Klebs-Loeffler organism. This method, which, briefly stated, involves the comparative study of selected characteristics in large numbers of individuals by exact and quantitative methods, has shed much light on anthropological and sociological problems. It was used first in bacteriology on a large scale by the Winslows in the study of the coccaceae, and has made possible a natural classification of the numerous members of this family. Since the Winslows'

* Received for publication July 12, 1912.

[1] *Systematic Relationships of the Coccaceae*, 1908.

first publication, several other articles have appeared, in which other bacteriological groups have been studied by this method. Notable among these researches are the further investigation by Winslow and Palmer[1] of the intestinal streptococci, Howe's[2] classification of the *B. coli* group, and Park and Krumwiede's[3] work on the human and bovine types of the tubercle bacillus.

The present study was originally suggested by a diphtheria epidemic at the Boston State Hospital in 1909, and was undertaken at the instance of Dr. E. E. Southard, pathologist to the Massachusetts state hospitals. The cultures have been obtained from the Boston and Danvers State Hospitals; the Bacteriological Laboratory of the Boston Board of Health, through the kindness of Dr. B. L. Arms and Miss E. M. Wade; from the Research Laboratories of the New York Board of Health; the Hoagland Laboratory, Brooklyn; the Chicago Board of Health; the Johns Hopkins Hospital; the New England Hospital for Women and Children, and from other scattered sources too numerous to mention. The writer has had throughout the personal assistance and interest of Professor Winslow, and is also indebted to Dr. Henry P. Frost, superintendent of the Boston State Hospital.

In the diphtheria group are usually included, in a loose way, the Klebs-Loeffler bacillus, Hofmann's bacillus, and the "diphtheroids," the last a collection of incompletely and unsystematically described forms, which includes the xerosis bacillus and all organisms bearing any morphological resemblance to the true diphtheria bacillus.

The first criterion by which bacilli were selected for the present study was necessarily morphological. All organisms which could be collected from throat cultures and pathological conditions, and which bore any morphological resemblance to the Klebs-Loeffler bacillus were worked out, provided that they were also gram-positive and non-motile.

The following tests were found most suitable and were adopted:

Morphology.—This comprised a study of 24-hour serum cultures, the presence of Neisser's granules, and the development of involution forms.

[1] *Jour. Infect. Dis.*, 1910, 7, p. 1.

[2] *Science*, N.S., 1912, 35, p. 225.

[3] *Jour. Med. Res.*, 1910, 18, p. 205.

The morphological classification of the diphtheria bacillus is admittedly a matter of dispute, not only on account of its pleomorphism but also because of the subjective element which enters into one's estimation of the frequency and value of the different forms. It was decided not to use Wesbrook's detailed classification in this work, as the object was not to diagnose for public health purposes, but to ascertain whether or not certain morphological types of culture could be correlated with other characteristics. The cultures therefore were grouped broadly on the basis of 24-hour growth on serum as to the predominating type of organism. Our experience agrees with that of Williams[1] that the morphological individuality of a culture is retained tenaciously under good conditions. It was found that all the strains collected could be divided into six morphological types, which formed the most satisfactory basis for a preliminary study of the group.

These types of culture may be defined as follows:

I. Cultures showing mostly slender curved bacilli of varying length, with metachromatic granules. The bacilli develop characteristic involution forms with swollen ends and irregularly staining protoplasm.

II. Cultures composed chiefly of long, curved, segmented, or barred bacilli, which often develop later into large bizarre "Indian-club" or "comma" forms.

III. Cultures in which solid and wedge-shaped bacilli predominate, although small granular and barred forms are also present and involution forms similar to those of type I appear later.

IV. Cultures showing small, thick forms, solid, barred, or wedge-shaped. These are smaller and show less variation in form than organisms of the preceding types, and do not stain as well. Neisser's granules are abundant but are small and indistinct. A marked peculiarity is the fusion of the bacilli in serum cultures at the end of several days into poorly staining masses, throughout which are scattered large, thick, intensely staining bacilli.

V. Cultures composed of large, thick, curved forms with regular, sharply outlined segments. These bacilli are larger and thicker than those of any of the foregoing classes except II, and they stain very clearly. Neisser's granules are numerous, large, and often of irregular shape. This type may develop the same involution forms as IV, but more often the bacilli remain separate, showing only an increase in size and more irregularity in staining.

VI. Cultures showing well-stained, usually straight bacilli, solid, wedge-shaped, or barred. These are shorter, and more uniform in appearance than those of type III. The 24-hour cultures often resemble closely type IV. Neisser's granules, however, are absent, and the organisms usually retain their original morphology and staining properties well, and do not develop the involution forms characteristic of the other types.

These morphological classes will be referred to by number.

[1] *Jour. Med. Res.*, 1902, 3, p. 83.

The photomicrographs, illustrating the six morphological types in the diphtheria group, are made from specimens from 24-hour serum cultures, stained with Loeffler's methylene blue; ×1,500.

FIG. 1, TYPE 1.

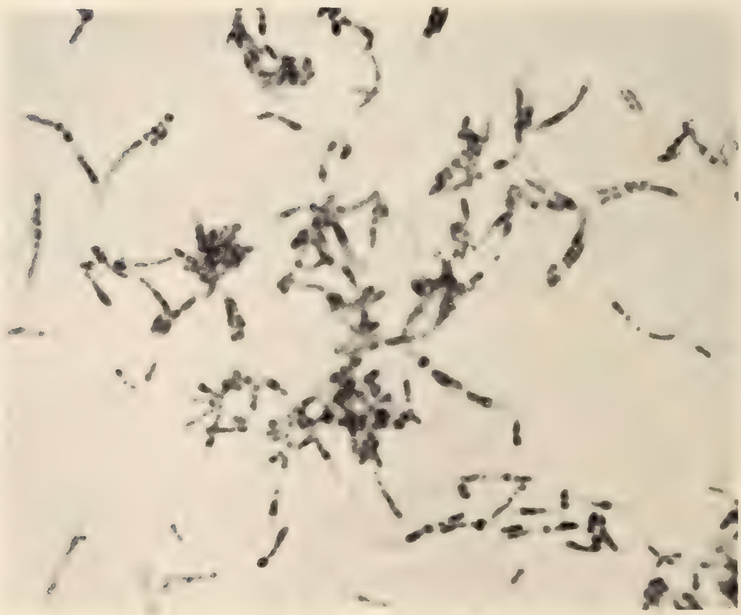

FIG. 2, TYPE 2.

Fig. 3, Type 3.

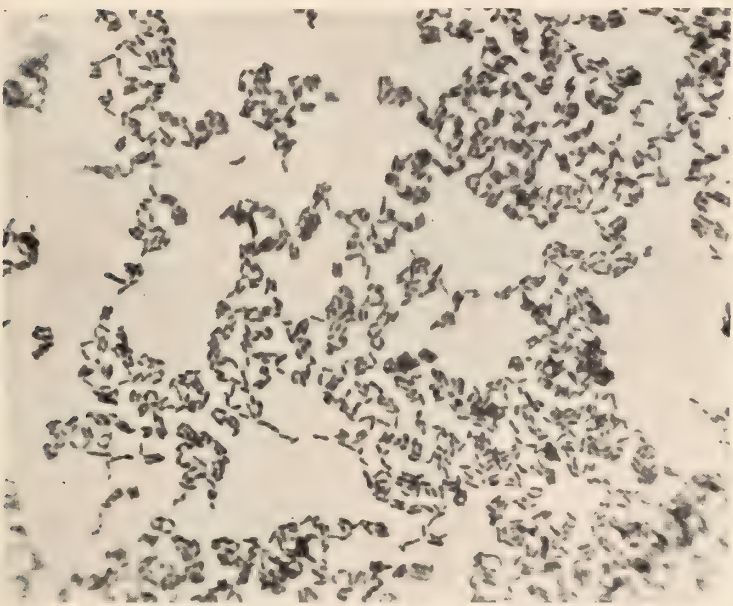

Fig. 4, Type 4.

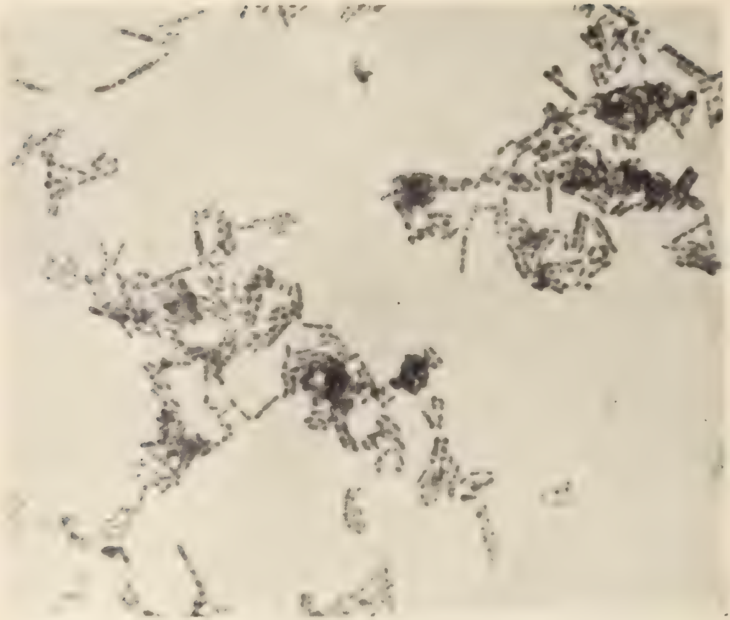

FIG. 5, TYPE 5.

FIG. 6, TYPE 6.

It must be emphasized in the beginning that this grouping can be only approximate, as every culture is a mixture of types; also, that the diagnosis of the type cannot be made with certainty in every case from a methylene blue stain of the 24-hour culture alone, but that the presence of granules and the development of involution forms must also be taken into account. This classification, however, does bring out important differences in cultures, which can be correlated with other characteristics.

Obviously, types I and II represent the usual granular and segmented forms of the diphtheria bacillus. Type III is closely related both to I and to IV. Types IV and V correspond to the common forms of "diphtheroids," and VI to Hofmann's bacillus.

Acid production.—The acid production was quantitatively determined by titration in one per cent dextrose, maltose, saccharose, dextrin, and glycerin broth. Dextrose was chosen as a representative of the glucose group, maltose and saccharose of the cane sugar group, dextrin of the cellulose group, and glycerin as a triacid alcohol. Although dextrin is a mixture and probably of somewhat variable composition, it seemed wise to use it in view of the large amount of work done on its fermentation by the diphtheria bacillus, and the varying results obtained by different observers. Merck's "highest purity" products and c.p. glycerin were used.

The sugars were added to the broth after the latter had been sterilized. A 10 per cent solution of the various carbohydrates in distilled water was heated 10 minutes in the Arnold sterilizer and added in appropriate amounts to sugar-free broth having a reaction of 0.5 per cent alkaline. The broth was then tubed out aseptically and incubated three days before use. Glycerin was added without sterilization.

A series of 10 organisms was titrated in each of the carbohydrates every other day for 20 days, to determine at what period, on the average, the maximum acidity occurred. Thereafter the cultures were titrated on these particular days. It was found that the maximum acidity for dextrose and saccharose occurred on the average on the 13th day, for maltose and dextrin on the eighth or ninth, and for glycerin on the 15th or 16th. The method of titration

was as follows: the tubes were put for five minutes in boiling water 5 c.c. of the broth were then removed with a graduated syringe, 45 c.c. of hot water added, and the mixture titrated immediately with $N/20$ NaOH; phenolphthalein being used as indicator. Two tubes were titrated for each strain, and their average taken. Two sterile blanks were put through with each lot, and the average of their acidity subtracted from that of the inoculated tubes. No culture which did not show a good growth was titrated, and the tests were repeated whenever there seemed to be reason to doubt their accuracy.

Fermentation reactions with several other substances were tried on a small scale, but were discontinued. Galactose and levulose gave results very similar to dextrose. Inulin and mannite were not decomposed by any strains studied.

The stability and uniformity of fermentation reactions are of vital importance for their use in classification. These points have been tested, first, on cultures of the same strain from different sources (Park No. 8 from three different places, No. 61 of the American Museum of Natural History, and No. 45 of the New York Health Department--the same strain); second, on different colonies from the same culture; and third, by repetition of the tests on different generations of the same culture; at intervals varying from a week to a year.

It has been found that there usually is qualitative uniformity of reaction, but considerable quantitative variation. In other words, cultures of the same strain from different sources, and of different colonies from the same culture show as a rule the same reaction. The exception to this rule is glycerin, in which, of two tubes put through at the same time, one occasionally may be alkaline, the other highly acid. In dextrose, maltose, and saccharose there is, on the other hand, a marked uniformity.

The fermentation reactions also remain permanent for a long period under laboratory cultivation, i.e., a strain does not easily lose fermentative powers or acquire new ones.

There are frequently, however, considerable differences in the amount of acid formed even by the same strain in quickly repeated tests, under identical conditions. These variations are shown with

glycerin, as has been noted above, and in a lesser degree with dextrin.

Virulence.—The virulence was tested by the subcutaneous inoculation of young guinea-pigs (150–250 gms.) with 0.5 per cent of their body weight of a 48-hour culture in sugar-free broth having an initial reaction of minus 0.5 per cent. Virulence was tested as soon as an organism had become accustomed to the medium and was growing well. No cultures were used in which growth was not fairly abundant. Only those strains were considered virulent which produced death of the animal with characteristic lesions within four days. Organisms giving merely a local reaction were classed as non-virulent.

Twenty-eight strains of diphtheroids were inoculated in the same manner as the diphtheria bacilli, and were found to be totally avirulent. The remainder were not tested. Twenty-one strains of Hofmann's bacillus were also tested for virulence, with negative results. No examples of Hamilton's[1] virulent pseudo-diphtheria bacillus have been found.

Toxin production.—Toxin production was not used as a routine procedure but as an aid in placing certain doubtful non-virulent bacilli. The method used was that of Rosenau, described in "The Immunity Unit for Standardizing Diphtheria Antitoxin."[2]

Chromogenesis.—Chromogenesis appears to be of some value, chiefly among the diphtheroids, though it is not as strikingly correlated with other characteristics as among the coccaccae. It was studied in serum cultures, as the colors were found to develop more characteristically on this than on other media. On agar the diphtheroids showed a white growth with sometimes a pink or yellow tinge. Contrary to experience with many organisms, potato did not prove satisfactory, as on it the diphtheroids formed a non-characteristic grayish-yellow growth with brown discoloration of the potato. Nährstoff gave the same results as plain agar. The chromogenesis varies somewhat with the alkalinity of the serum, being most marked when it is alkaline. That used in this work had a uniform reaction of 0.8 per cent alkaline.

[1] *Jour. Infect. Dis.*, 1904, 1, p. 690.

[2] Rosenau, M. J., "The Immunity Unit for Standardizing Diphtheria Antitoxin," *Hygienic Laboratory of Public Health and Marine Hospital Service*, Bull. 21, April, 1905.

The following color-scheme expresses the shades found in serum cultures, and has therefore been adopted for correlation of chromogenesis with other characteristics: colorless, white, gray, yellow-white, yellow, and salmon-pink. The color was noted at the end of four days' growth at 37.5°.

Vigor of growth.—The vigor of the growth was noted in 24-hour and four-day cultures. Among the diphtheria bacilli, the differences were not great after the strains had become accustomed to the medium. A few strains, however, remained persistently feeble, and soon died out. Two varieties of diphtheroids grew quickly and abundantly, a third was characterized by slow and scanty growth. The degrees of surface growth have been classified as scanty, medium, and heavy.

Fixation of complement.—This part of the work will form the subject of a separate report.

Two hundred and ninety-five strains were examined, divided among the morphological classes as follows: type I, 112; II, 52; III, 25; IV, 48; V, 32; VI, 26. As a rule only one strain was isolated from each source and one set of titrations made on each strain.

RESULTS.

A preliminary orientation is obtained by a glance at Table 1, which shows the qualitative relationships between the six morphological groups and the other characters studied.

TABLE 1.

RELATIONSHIP OF MORPHOLOGICAL TYPES TO OTHER CHARACTERS.

Type	Acid Production in					Virulence	Toxin Production	Chromogenesis	Vigor of Growth
	Dextrose	Maltose	Glycerin	Saccharose	Dextrin				
I	+	+	+	−	±	+	+	Gray	+
II	+	+	+	−	±	±	+	Gray	+
III	+	±	−	±	−	∓	±	Gray, often with pink tinge	+
IV	+	−	−	+	−	−	−	Salmon-pink	++
V	+	+	±	±	−	−	−	Yellow or yellow-white	+
VI	−	−	−	−	−	−	−	White	+

The sign ± indicates that positive and negative results were obtained in about equal proportions; the sign ∓ that while the results in general were negative some positive ones were obtained.

The most important deductions from the table are as follows: there is a decrease in fermentative powers and virulence in passing from morphological groups I to VI; the chromogenic cultures belong to IV and V; and group III is intermediate in character in fermentation reactions, virulence, and chromogenesis.

The curves of acidity in the five carbohydrates for all the organisms investigated may also serve as an introduction to a more extensive study.

The curve in dextrose broth (Chart 1) shows two groups, one non-acid, the other acid-forming with its mode at 4.0–4.5 per cent. In the first group are morphological type VI, a few non-virulent bacilli of I, II, and III, and a few bacilli of type IV. In the acid group are the majority of types I to V.

In maltose broth also (Chart 1) two groups, an acid and a non-acid, are apparent. The non-acid group comprises types IV and VI; the acid group contains I, II, and V. Type III is represented in both groups.

In saccharose broth (Chart 1) the curve shows two groups, one alkaline, neutral, and weakly acid, the other highly acid. Classes I, II, V, and VI and a part of III form the first group, while the latter is composed of IV and some of III.

The curves (Chart 2) in dextrin and glycerin show all organisms varying about one mode, the neutral point.

The relationship of the morphological groups to each of the characters studied will be analyzed in detail.

Correlations between morphological groups and fermentation reactions.—In dextrose broth (Table 2) the close similarity of

TABLE 2.

Correlation between Morphological Types and Fermentation of Dextrose. Acid Produced (Percentage Normal).

Types	Number of Cultures of Each Type							
	Alkaline −0.0	0.0–1.0	1.0–2.0	2.0–3.0	3.0–4.0	4.0–5.0	5.0–6.0	Above 6.0
I	0	4	2	5	34	33	7	7
II	0	2	2	5	15	17	10	5
III	0	2	5	3	5	7	2	2
IV	5	8	7	8	5	6	3	6
V	0	3	7	4	7	11	5	1
VI	25	3	0	0	0	0	0	0

groups I and II is apparent, both having their modes at 3.0–5.0 per cent. Groups III and V resemble the first two. Group IV

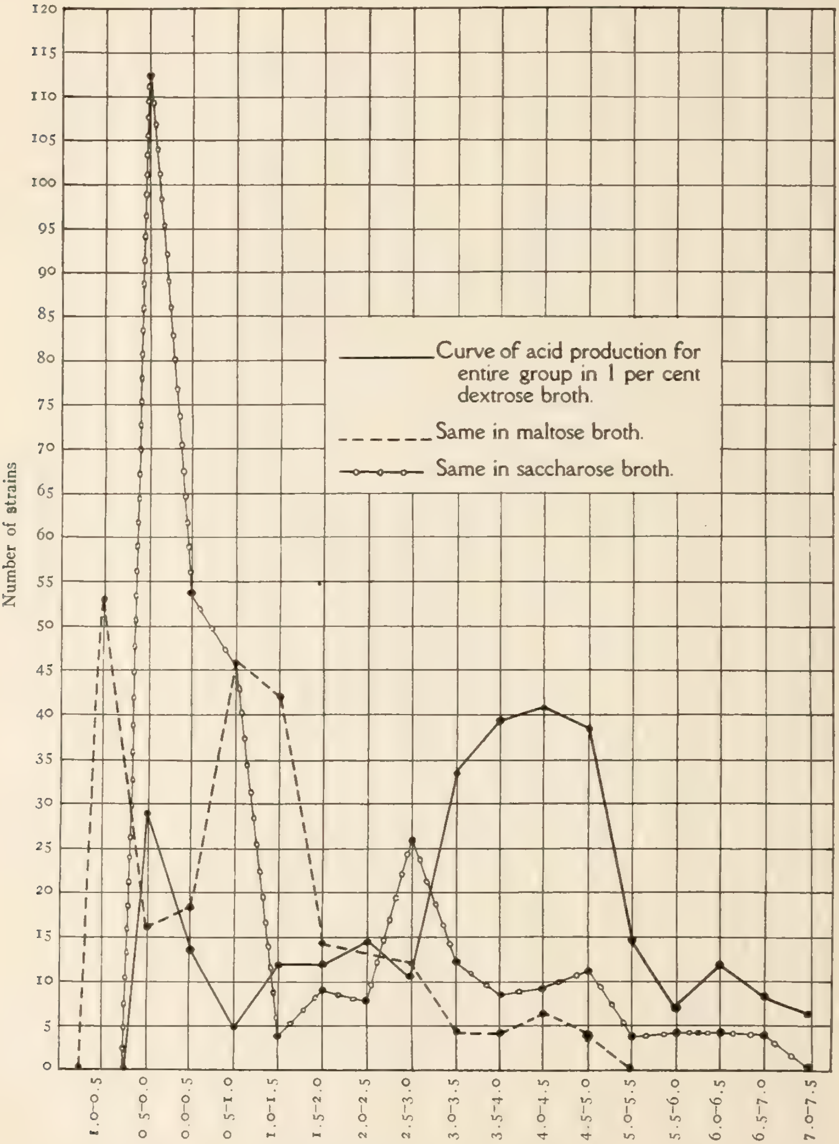

CHART 1.

shows a wide range from alkalinity to high acidities. In VI all cultures are below 1 per cent acidity.

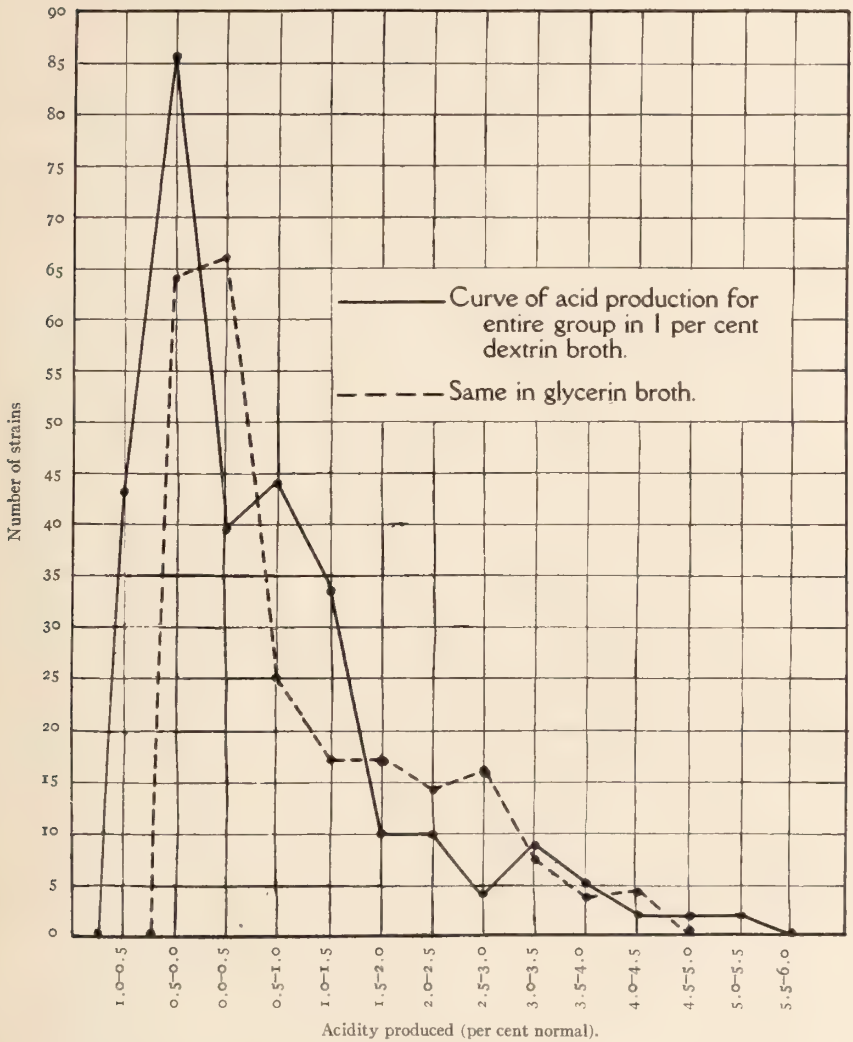

Chart 2.

No special stress can be laid on quantitative results in the fermentation of glycerin, because of the great variability of reaction to it. Table 3, however, does bring out two important points, i.e.,

that groups IV and VI do not acidify glycerin, while V has considerable fermentative powers.

TABLE 3.

CORRELATION BETWEEN MORPHOLOGICAL TYPES AND FERMENTATION OF GLYCERIN. ACID PRODUCED (PERCENTAGE NORMAL).

Types	Number of Cultures of Each Type					
	Alkaline −0.0	0.0–1.0	1.0–2.0	2.0–3.0	3.0–4.0	Above 4.0
I	19	36	20	19	4	0
II	2	16	9	8	0	0
III	4	6	2	0	0	0
IV	23	16	0	0	0	0
V	5	10	3	3	5	1
VI	15	8	0	0	0	0

Analysis of the results in maltose broth (Table 4) again brings out similarity between groups I and II and slight fermentative powers in III and VI. Thirty-nine, or 81 per cent of type IV are either alkaline or below 1 per cent acidity. Members of V, on the other hand, are strong acid-producers.

TABLE 4.

CORRELATION BETWEEN MORPHOLOGICAL TYPES AND FERMENTATION OF MALTOSE. ACID PRODUCED (PERCENTAGE NORMAL).

Types	Number of Cultures of Each Type					
	Alkaline −0.0	0.0–1.0	1.0–2.0	2.0–3.0	3.0–4.0	4.0–5.0
I	0	33	37	4	1	0
II	3	14	15	3	0	0
III	5	3	1	0	0	1
IV	27	12	2	5	1	1
V	2	5	3	3	6	9
VI	11	9	1	0	0	0

In saccharose broth (Table 5) groups I, II, and VI are gathered about the neutral point. Of groups III and V, half are below

TABLE 5.

CORRELATION BETWEEN MORPHOLOGICAL TYPES AND FERMENTATION OF SACCHAROSE. ACID PRODUCED.

Types	Number of Cultures of Each Type								
	Alkaline −0.0	0.0–1.0	1.0–2.0	2.0–3.0	3.0–4.0	4.0–5.0	5.0–6.0	6.0–7.0	Above 7.0
I	43	35	1	1	5	1	0	0	0
II	45	18	1	1	0	0	0	0	0
III	10	9	3	7	6	1	1	2	0
IV	0	3	4	9	23	8	9	5	3
V	19	7	6	2	3	2	1	0	0
VI	16	9	0	1	0	0	0	0	0

1 per cent acidity, the other half range to high acidities. Group IV is composed of strong acid-producers.

Dextrin (Table 6) is acted on to the same extent by types I and II, but is not decomposed by the last four.

TABLE 6.

CORRELATION BETWEEN MORPHOLOGICAL TYPES AND FERMENTATION OF DEXTRIN. ACID PRODUCED (PERCENTAGE NORMAL).

Types	Number of Cultures of Each Type						
	Alkaline —0.0	0.0–1.0	1.0–2.0	2.0–3.0	3.0–4.0	4.0–5.0	5.0–6.0
I........	16	32	30	11	8	1	0
II........	5	25	10	4	3	0	1
III........	6	16	4	0	1	1	0
IV........	27	12	3	0	0	0	0
V........	12	23	0	0	0	0	0
VI........	16	5	0	0	0	0	0

Correlations between morphological types and virulence.—A study of the relationship between morphological types and virulence for guinea-pigs brings out the fact that of type I, 57 cultures or 61.3 per cent were virulent and 36 or 38.7 per cent non-virulent; while of type II, 17 cultures or 33.3 per cent were virulent, and 34 or 66.6 per cent non-virulent. Type III showed 18 non-virulent and only one virulent culture.

TABLE 7.

CORRELATION BETWEEN MORPHOLOGICAL TYPES AND VIRULENCE FOR GUINEA-PIGS.

	Number of Cultures of Each Type					
	I	II	III	IV	V	VI
Virulent.........	57	17	1	0	0	0
Non-virulent.....	36	34	18	18 (tested)	10 (tested)	21 (tested)

Six cultures of class IV and three of V were tested for toxin production. Injections of the filtrate from two strains of class IV produced local induration in doses of 0.1 c.c., from another strain in a dose of 1.0 c.c. All of the animals recovered. Filtrates of the other six cultures were without effect in doses of 1 c.c.

Correlation between morphological types and chromogenesis.—The great majority (90 per cent each) of cultures of types I and II were gray. There were a few colorless, white, and yellow-white variants,

and a few (3 per cent each) showing a salmon-pink tinge. The proportion of the latter rose in type III to 23 per cent. In IV salmon-pink was the prevailing color (83 per cent), the remainder of the group being yellow, yellow-white, or white. Type V is represented in all classes except gray from colorless to yellow, but, as will be seen later, it is made up of two subdivisions, slowly and scantily growing strains, showing a colorless or white growth and differing slightly in fermentation reactions from the abundantly growing yellow and yellow-white cultures.

Most of the members of type VI gave a white or yellow-white growth.

TABLE 8.

CORRELATION BETWEEN MORPHOLOGICAL TYPES AND CHROMOGENESIS.

Type	Number of Cultures in Each Class					
	Colorless	White	Gray	Yellow-white	Yellow	Salmon-pink
I	3	1	101	3	0	4 (tinge)
II	1	0	46	3	0	2 (tinge)
III	1	0	16	3	0	6
IV	0	1	0	4	3	40
V	7	7	0	8	10	0
VI	1	15	0	8	0	2

Correlation between morphology and vigor of growth.—This is of limited value because, as has been said, a strain which grew poorly at first may later become accustomed to the medium, and flourish abundantly. The vigor of growth, therefore, has been estimated, not from the first culture alone, but also from successive replants.

Table 9, however, shows several important facts. The majority of cultures of type III grew heavily from the start. Strains of

TABLE 9.

CORRELATION BETWEEN MORPHOLOGY AND VIGOR OF GROWTH.
MORPHOLOGICAL TYPES.

Surface Growth	Numbers of Cultures of Each Type					
	I	II	III	IV	V	VI
Scanty	35	18	2	0	13	8
Medium	44	16	6	3	1	3
Heavy	33	18	17	45	18	15

group IV all grew abundantly. In group V, two subdivisions are again apparent, as in Table 8, the scantily growing cultures being

colorless or white, and the heavily growing strains yellow or yellow-white.

The numbers of groups I, II, and VI in the different classes of growth are not especially significant.

From the preceding analysis, it is clear that there are two large subdivisions in the diphtheria group. The first is composed of slender, curved, granular, or segmented bacilli (types I and II) which vary greatly in morphology and develop characteristic involution forms. The members of this subgroup form acid in dextrose and maltose, and in about half the cases in glycerin and dextrin also, but they do not act on saccharose. The prevailing color of these cultures is gray. In man and in guinea-pigs many members of this group produce characteristic lesions due to a specific toxin.

The members of the second subdivision, which includes types IV, V, and VI, show a marked morphological resemblance to each other, and a lesser resemblance to members of the first subgroup. They are thicker and more regular than the latter and do not develop the same involution forms; they are non-virulent for guinea-pigs and do not form the toxin characteristic of the first group. The possibility of a pathogenic action for man will be touched on later. They show differences in fermentation reactions, agreeing only in their inability to acidify dextrin. Chromogenic cultures (yellow and salmon-pink) are frequent. All degrees of growth, from scanty to very heavy, are represented. The correlated differences in fermentation reactions, chromogenesis, and vigor of growth permit a further subdivision of this group into species.

There are also a few strains which, while coinciding in most properties with the first group, show features characteristic of the second. These cultures are represented by morphological type III.

This primary subdivision is a justification by the biometrical method of the current broad classification of the diphtheria group into the diphtheria bacillus and the "diphtheroid" bacilli.

Each of these groups will be considered in detail.

First Subgroup, the Diphtheria Bacillus (Morphological Types I, II, and III).

A glance at the table of relationship between morphology and the source of the culture, i.e., whether from a clinical case, a carrier,

or from an unusual situation or lesion, shows that, while types I and II were represented in approximately equal numbers among clinical cases and carriers, the bacilli from unusual situations were, with one exception, of type II.

TABLE 10.

CORRELATION BETWEEN MORPHOLOGY AND HABITAT OR LESION.

Type	Pharyngeal Diphtheria	Nasal Diphtheria	Carriers	Other Situations
I	41	6	20	1-sinus of elbow joint
II	37	2	18	2-eye 1-abscess of breast 1-sputum asthma 1-vagina 1-nose, with ozena bacillus
III	5	0	16	0

Cultures from unusual situations include two from the eye, and one each from a sinus leading to the elbow joint, an abscess of the breast, the sputum from a case of asthma, the vagina, and the nose, associated with ozena bacillus. The majority of cultures of type III came from carriers, and only a small proportion from clinical cases (16 and 5, respectively).

In other words, diphtheria bacilli from parts of the body other than the throat and nose are more often of the segmented than of the granular type, and the small granular and solid forms are not often found in clinical diphtheria.

Correlations between sources of cultures and their virulence do not yield any new information. Of cultures taken from cases which the records sent to the Health Department showed to be cases of clinical diphtheria, 40 were virulent and 29 avirulent for guinea-pigs. Of cultures from carriers, mostly school-children, 13 were virulent and 37 avirulent. Cultures for release showed one virulent and three non-virulent. From cases of nasal diphtheria seven strains were virulent, and one non-virulent.

Five non-virulent strains were obtained from the nose, throat, and ears of scarlet fever patients. Two virulent cultures were obtained from the eye.

Cultures from unusual situations (abscess of the breast, sputum in a case of asthma, vagina) were non-virulent, with one exception—

a strain from a sinus following a fracture. The correlation of virulence with the three morphological types has already been devel-

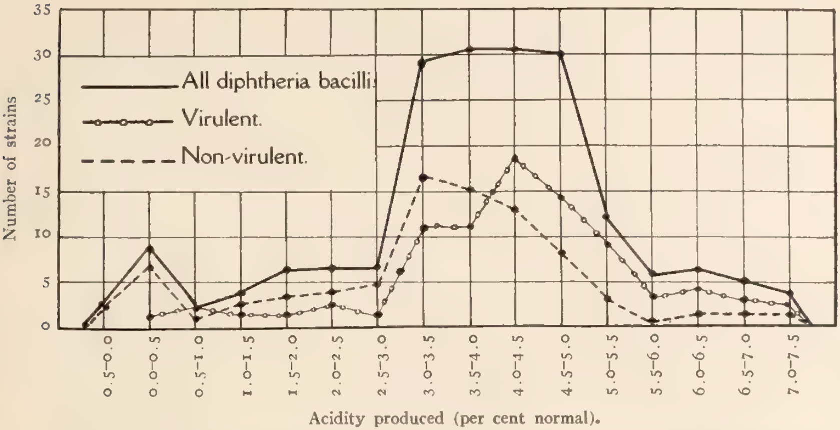

CHART 3.—Curves of acid production for types I, II, and III (diphtheria bacilli) in one per cent dextrose broth.

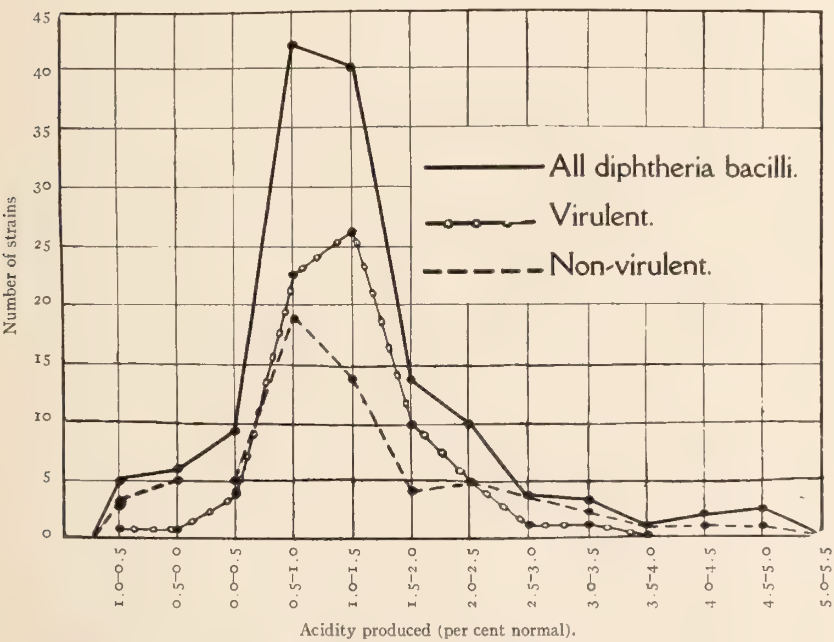

CHART 4.—Curves of acid production for types I, II, and III (diphtheria bacilli) in one per cent maltose broth.

oped. Of the granular cultures, the majority are virulent, while of the segmented two-thirds are non-virulent. The small granular type are almost all non-virulent.

The curves of acid formation for types I, II, and III (Charts 3 to 7) will be considered next. Those in maltose, saccharose, glycerin, and dextrin show only one mode. In dextrose broth, there

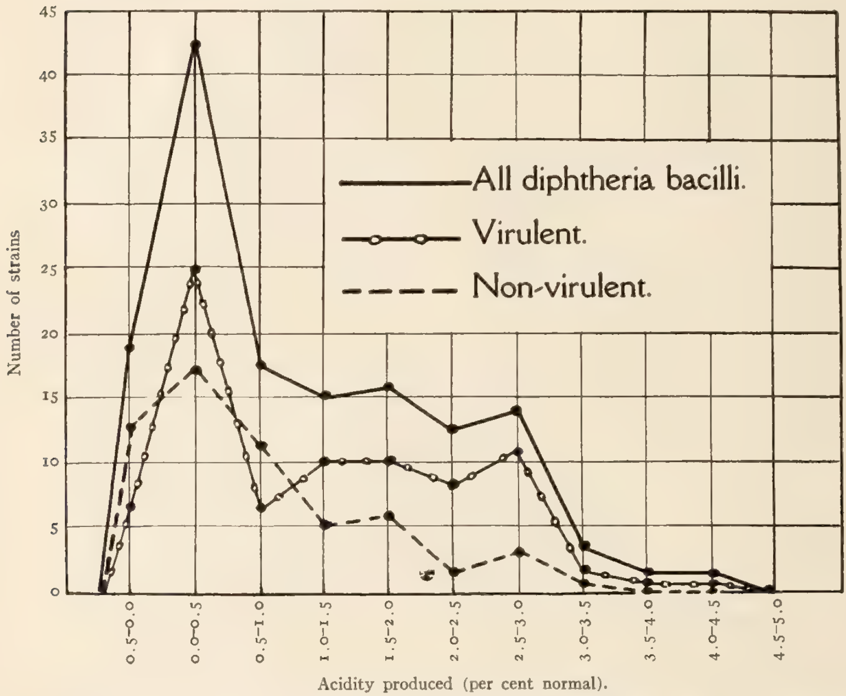

CHART 5.—Curves of acid production for types I, II, and III (diphtheria bacilli) in one per cent glycerin broth.

are two modes, a slight one at 0.0–0.5, and the chief one at 3.0–5.0 per cent. Plotting the curves for virulent and non-virulent diphtheria bacilli separately brings out slight differences between the two groups. In dextrose, maltose, and dextrin, the mode for the non-virulent series falls in each case at a lower degree of acidity than that for the virulent, while in saccharose most of the bacilli forming acid are non-virulent.

Analysis of the curve in dextrose broth shows that non-virulent

strains are chiefly responsible for the low acid group (nine non-virulent and one virulent). The mode for the high-acid group

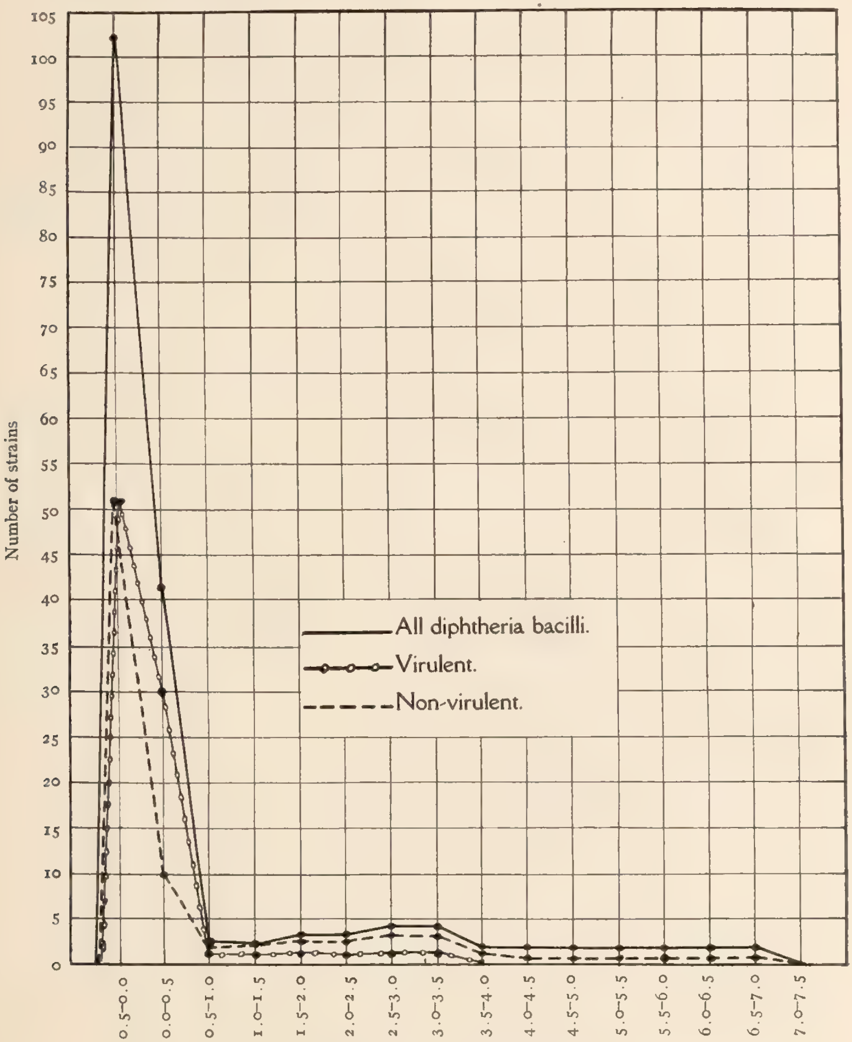

Chart 6.—Curves of acid production for types I, II, and III (diphtheria bacilli) in one per cent saccharose broth.

falls at 3.0–3.5 per cent for the non-virulent organisms; at 4.0–4.5 per cent for the virulent.

In maltose broth, the mode for the non-virulent strains is at 0.5–1.0, for the virulent at 1.0–1.5 per cent.

In dextrin broth, both virulent and non-virulent bacilli are represented to the same extent in the alkaline group, but the second mode for the non-virulent organisms is at 0.0–0.5 per cent; for the virulent at 1.0–1.5 per cent. Among the non-virulent there are no strains giving an acidity above 3.5 per cent.

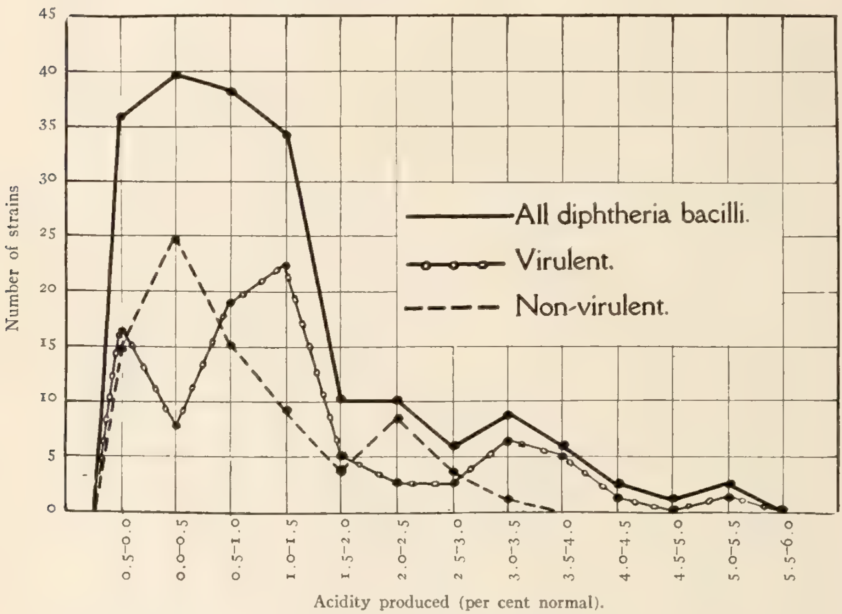

CHART 7.—Curve of acid production for types I, II, and III (diphtheria bacilli) in one per cent dextrin broth.

In glycerin broth, there is no difference between virulent and avirulent groups, the mode for both falling at 0.0–0.5 plus.

It has been a debated point whether or not differences, morphological or biochemical, could be demonstrated between virulent and avirulent diphtheria bacilli. These results show that when a long series is examined, distinct though small differences do appear in the groups as a whole, both in morphology and in fermentative powers. In the virulent group, granular bacilli predominate, forming 76 per cent; segmented organisms form 22 per cent, and small granular and solid types about one per cent. In the avirulent

group, granular and segmented forms are present in about equal proportions (the former 40 per cent, the latter 38 per cent), while 21 per cent of the group is made up of small granular and solid types. The virulent organisms have slightly greater action on dextrose, maltose, and dextrin. On the other hand, non-virulent organisms predominate among the saccharose fermenters.

It is apparent, however, that virulent and non-virulent forms are very closely related, and that the practice, common among medical workers, of limiting the term "true diphtheria bacillus" to the virulent forms, and using the expression "diphtheria-like" bacilli for the non-virulent, is in reality confusing and not justified biologically. In view of the ease with which virulence is known to be modified in other bacteria, it seems questionable whether it should be considered as having more than varietal significance.

The aberrant character of type III in relation to I and II and its similarity to type IV has been brought out. The resemblance is shown by its heavy growth, often with a pink tinge, its relatively slighter action on dextrose, maltose, and glycerin, on the one hand, and, on the other, its frequent fermentation of saccharose. These strains should perhaps be classed with diphtheroids rather than with diphtheria bacilli, but it is clear that no sharp line can be drawn biologically between these organisms and the typical diphtheria bacillus on the one side and group IV of the diphtheroids on the other. The typical forms of diphtheria and diphtheroid bacilli are of course widely separated, but there have been found in the course of this study a number of cultures which showed one or more of the properties considered characteristic of diphtheroids (e.g., chromogenesis, or fermentation of saccharose) while in other properties resembling the usual diphtheria bacillus. One of these strains produced sufficient toxin to kill a guinea-pig within two days when injected in a dose of 0.1 c.c.

SECOND SUBGROUP. THE SO-CALLED DIPHTHEROIDS AND HOFMANN'S BACILLI. (MORPHOLOGICAL TYPES IV, V, AND VI).

The members of this subgroup present certain general characteristics. Their morphology is distinctive. All that were collected during this work were gram-positive and non-motile. Neither the

gram-negative diphtheroid bacilli described by Hamilton[1] nor the motile forms described by DeWitt[2] have been observed in this study.

Neisser's granules were as a rule present in types IV and V, only five strains failing to produce them at the end of four days. In a number of cases, however, they developed late, being absent in 24-hour cultures, but numerous at the end of four days. Differences in the size and form of the granules were observed in the two types, as has been mentioned. No granules were found in type VI.

On serum, the majority of the strains produced heavy salmon-pink, yellow, or yellow-white growths. Colonies of the primary growth frequently were invisible for several days, but replants usually grew quickly and abundantly. Some strains, however, showed only a slow, scanty, colorless growth.

All the organisms were isolated from the human body, where these bacilli are not infrequently found in a variety of situations, both in normal and in pathological conditions. They probably would be found more often if routine cultures were examined again after the lapse of several days or a week.

The characteristics on which further subdivisions of this group are based are fermentation reactions, vigor of growth, and chromogenesis.

The curves of acid-formation in dextrose, maltose, and saccharose are alone significant, as dextrin is not acted upon by any of the organisms and glycerin only by a few of type V.

In dextrose broth (Chart 8), two groups are apparent, one non-acid, the other highly acid. Between these there are numerous intergrading forms. The first group comprises type VI, and a few strains mostly of type IV, the second, the majority of IV and V.

The curve in maltose broth (Chart 8) likewise shows two groups: an alkaline and faintly acid group, and an acid group, with its mode at 2.0–2.5 per cent. In the first and largest group are classes III and most of IV; in the second class V and a few of IV.

The curve in saccharose broth (Chart 8) has two sharply defined modes, one alkaline, the other at 3.0–3.5 per cent acid. Around the first mode are gathered types VI and V, while around the second is type IV.

[1] *Jour. Infect. Dis.*, 1907, 4, p. 326. [2] *Ibid.*, 1912, 10, p. 36.

A study of the relation of surface growth and chromogenesis to these curves helps to bring out the grouping. In dextrose broth 10, or 50 per cent, of the poorly growing strains are either alkaline or below 1 per cent acidity (Table 11). Of the heavily growing

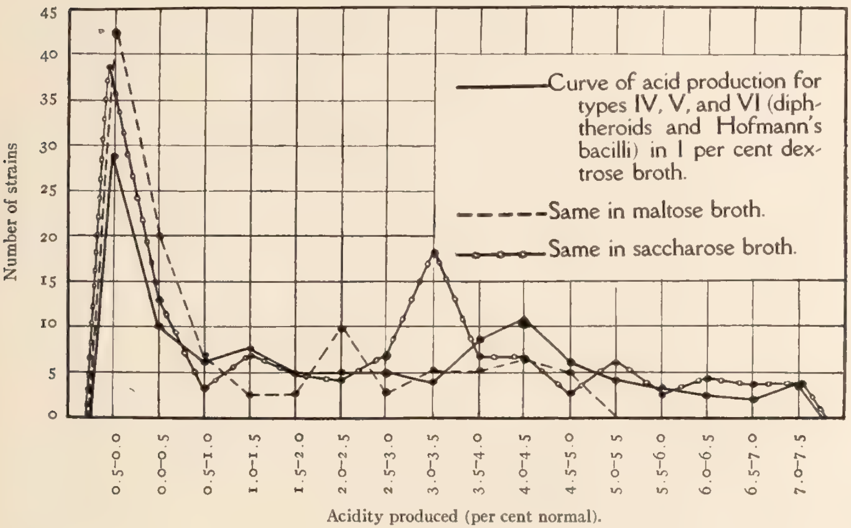

CHART 8.

cultures, 32, or 40 per cent, fall below 1 per cent and 29, or 36 per cent, above 3 per cent acidity. The majority of the low-acid, heavily growing cultures belong to type VI, which shows wide variation in

TABLE 11.

CORRELATION BETWEEN VIGOR OF GROWTH AND FERMENTATION OF DEXTROSE. ACID PRODUCED (PERCENTAGE NORMAL).

	Number of Cultures in Each Class					
	Alkaline — 0.0	0.0–1.0	1.0–2.0	2.0–3.0	3.0–4.0	Above 4.0
Scanty	9	1	5	2	2	1
Medium	2	0	1	0	2	3
Heavy	25	7	8	11	7	22

abundance of growth; the remainder belong to type IV. The heavily growing, high-acid group is composed of chromogenic cultures, chiefly yellow and salmon-pink, of types IV and V.

Correlation between degrees of surface growth and fermentation of maltose (Table 12) shows that 16, or 75 per cent, of the scantily growing forms are below 1 per cent acidity, and also 50, or 74 per cent, of the heavily growing strains. Here again, the scantily growing organisms belong to type VI, the heavily growing to types IV and VI. The acid-producers are of type V.

TABLE 12.

CORRELATION BETWEEN VIGOR OF GROWTH AND FERMENTATION OF MALTOSE. ACIDITY PRODUCED (PERCENTAGE NORMAL).

	Numbers of Cultures in Each Class					
	Alkaline −0.0	0.0–1.0	1.0–2.0	2.0–3.0	3.0–4.0	Above 4.0
Scanty	10	6	1	0	3	0
Medium	2	0	0	1	1	3
Heavy	46	4	3	6	4	4

The table of relationship between surface growth and fermentation of saccharose (Table 13) shows that 11 of the poorly growing strains are below 1 per cent, and only three above 2 per cent acidity. The heavily growing organisms are gathered at the two ends of the scale. The scantily growing non-acid strains are, as previously, of type VI, while the heavily growing non-acid group is composed of types V and VI. The highly acid group is formed by type IV.

TABLE 13.

CORRELATION BETWEEN VIGOR OF GROWTH AND FERMENTATION OF SACCHAROSE. ACID PRODUCED (PERCENTAGE NORMAL).

	Number of Cultures in Each Class					
	Alkaline −0.0	0.0–1.0	1.0–2.0	2.0–3.0	3.0–4.0	Above 4.0
Scanty	9	2	5	0	2	1
Medium	6	3	1	0	1	[illegible]
Heavy	27	5	5	11	18	22

Taking up chromogenesis more in detail, it is seen from Table 14, that nearly all of the colorless strains grew poorly, while the yellow-white, yellow, and salmon-pink cultures grew abundantly. The white strains were intermediate in position.

glycerin, as has been noted above, and in a lesser degree with dextrin.

Virulence.—The virulence was tested by the subcutaneous inoculation of young guinea-pigs (150–250 gms.) with 0.5 per cent of their body weight of a 48-hour culture in sugar-free broth having an initial reaction of minus 0.5 per cent. Virulence was tested as soon as an organism had become accustomed to the medium and was growing well. No cultures were used in which growth was not fairly abundant. Only those strains were considered virulent which produced death of the animal with characteristic lesions within four days. Organisms giving merely a local reaction were classed as non-virulent.

Twenty-eight strains of diphtheroids were inoculated in the same manner as the diphtheria bacilli, and were found to be totally avirulent. The remainder were not tested. Twenty-one strains of Hofmann's bacillus were also tested for virulence, with negative results. No examples of Hamilton's[1] virulent pseudo-diphtheria bacillus have been found.

Toxin production.—Toxin production was not used as a routine procedure but as an aid in placing certain doubtful non-virulent bacilli. The method used was that of Rosenau, described in "The Immunity Unit for Standardizing Diphtheria Antitoxin."[2]

Chromogenesis.—Chromogenesis appears to be of some value, chiefly among the diphtheroids, though it is not as strikingly correlated with other characteristics as among the coccaceae. It was studied in serum cultures, as the colors were found to develop more characteristically on this than on other media. On agar the diphtheroids showed a white growth with sometimes a pink or yellow tinge. Contrary to experience with many organisms, potato did not prove satisfactory, as on it the diphtheroids formed a non-characteristic grayish-yellow growth with brown discoloration of the potato. Nährstoff gave the same results as plain agar. The chromogenesis varies somewhat with the alkalinity of the serum, being most marked when it is alkaline. That used in this work had a uniform reaction of 0.8 per cent alkaline.

[1] *Jour. Infect. Dis.*, 1904, 1, p. 690.

[2] Rosenau, M. J., "The Immunity Unit for Standardizing Diphtheria Antitoxin," *Hygienic Laboratory of Public Health and Marine Hospital Service*, Bull. 21, April, 1905.

The following color-scheme expresses the shades found in serum cultures, and has therefore been adopted for correlation of chromogenesis with other characteristics: colorless, white, gray, yellow-white, yellow, and salmon-pink. The color was noted at the end of four days' growth at 37.5°.

Vigor of growth.—The vigor of the growth was noted in 24-hour and four-day cultures. Among the diphtheria bacilli, the differences were not great after the strains had become accustomed to the medium. A few strains, however, remained persistently feeble, and soon died out. Two varieties of diphtheroids grew quickly and abundantly, a third was characterized by slow and scanty growth. The degrees of surface growth have been classified as scanty, medium, and heavy.

Fixation of complement.—This part of the work will form the subject of a separate report.

Two hundred and ninety-five strains were examined, divided among the morphological classes as follows: type I, 112; II, 52; III, 25; IV, 48; V, 32; VI, 26. As a rule only one strain was isolated from each source and one set of titrations made on each strain.

RESULTS.

A preliminary orientation is obtained by a glance at Table 1, which shows the qualitative relationships between the six morphological groups and the other characters studied.

TABLE 1.
RELATIONSHIP OF MORPHOLOGICAL TYPES TO OTHER CHARACTERS.

Type	Acid Production in					Virulence	Toxin Production	Chromogenesis	Vigor of Growth
	Dextrose	Maltose	Glycerin	Saccharose	Dextrin				
I	+	+	+	−	±	+	+	Gray	+
II	+	+	+	−	±	±	+	Gray	+
III......	+	±	−	±	−	∓	±	Gray, often with pink tinge	+
IV...	+	−	−	+	−	−	−	Salmon-pink	++
V...	+	+	±	±	−	−	−	Yellow or yellow-white	+
VI......	−	−	−	−	−	−	−	White	+

The sign ± indicates that positive and negative results were obtained in about equal proportions; the sign ∓ that while the results in general were negative some positive ones were obtained.

The most important deductions from the table are as follows: there is a decrease in fermentative powers and virulence in passing from morphological groups I to VI; the chromogenic cultures belong to IV and V; and group III is intermediate in character in fermentation reactions, virulence, and chromogenesis.

The curves of acidity in the five carbohydrates for all the organisms investigated may also serve as an introduction to a more extensive study.

The curve in dextrose broth (Chart 1) shows two groups, one non-acid, the other acid-forming with its mode at 4.0–4.5 per cent. In the first group are morphological type VI, a few non-virulent bacilli of I, II, and III, and a few bacilli of type IV. In the acid group are the majority of types I to V.

In maltose broth also (Chart 1) two groups, an acid and a non-acid, are apparent. The non-acid group comprises types IV and VI; the acid group contains I, II, and V. Type III is represented in both groups.

In saccharose broth (Chart 1) the curve shows two groups, one alkaline, neutral, and weakly acid, the other highly acid. Classes I, II, V, and VI and a part of III form the first group, while the latter is composed of IV and some of III.

The curves (Chart 2) in dextrin and glycerin show all organisms varying about one mode, the neutral point.

The relationship of the morphological groups to each of the characters studied will be analyzed in detail.

Correlations between morphological groups and fermentation reactions.—In dextrose broth (Table 2) the close similarity of

TABLE 2.

CORRELATION BETWEEN MORPHOLOGICAL TYPES AND FERMENTATION OF DEXTROSE. ACID PRODUCED (PERCENTAGE NORMAL).

Types	Number of Cultures of Each Type							
	Alkaline −0.0	0.0–1.0	1.0–2.0	2.0–3.0	3.0–4.0	4.0–5.0	5.0–6.0	Above 6.0
I	0	4	2	5	34	33	7	7
II	0	2	2	5	15	17	10	5
III	0	2	5	3	5	7	2	2
IV	5	8	7	8	5	6	3	6
V	0	3	7	4	7	11	5	1
VI	25	3	0	0	0	0	0	0

groups I and II is apparent, both having their modes at 3.0–5.0 per cent. Groups III and V resemble the first two. Group IV

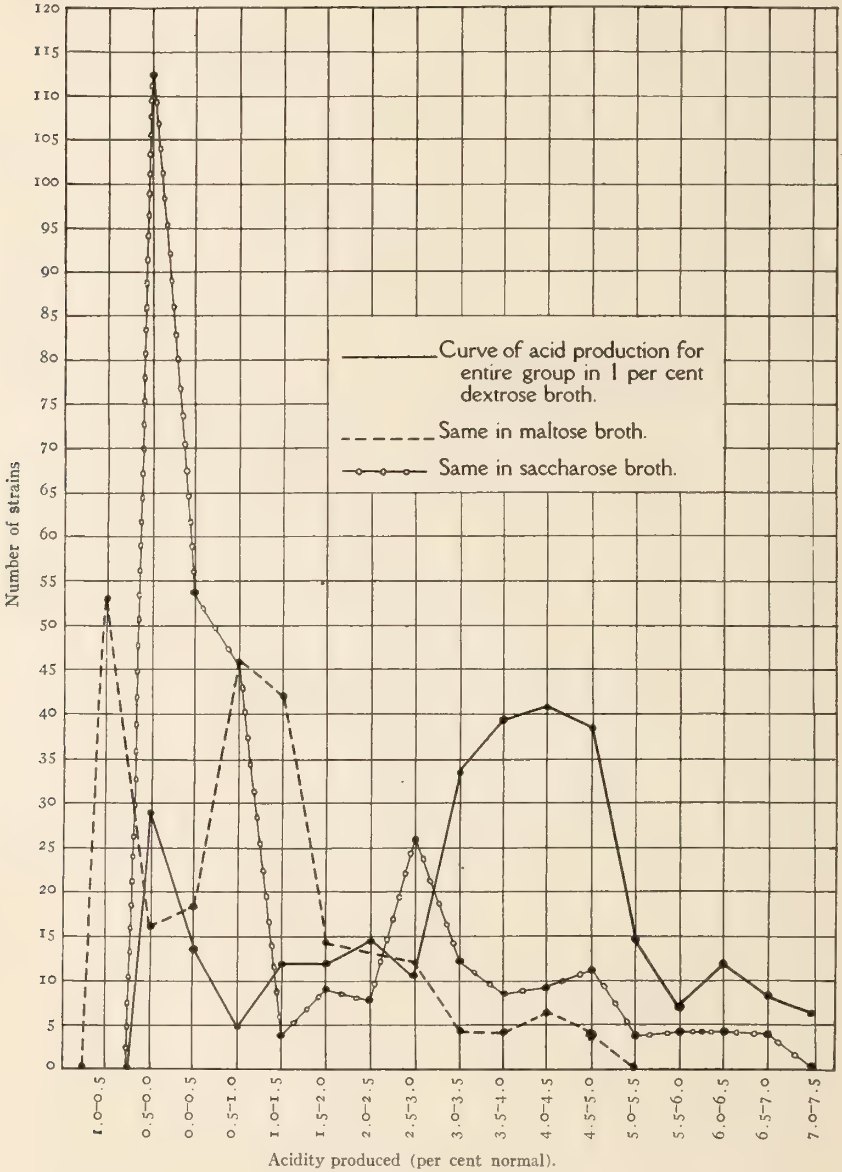

CHART 1.

shows a wide range from alkalinity to high acidities. In VI all cultures are below 1 per cent acidity.

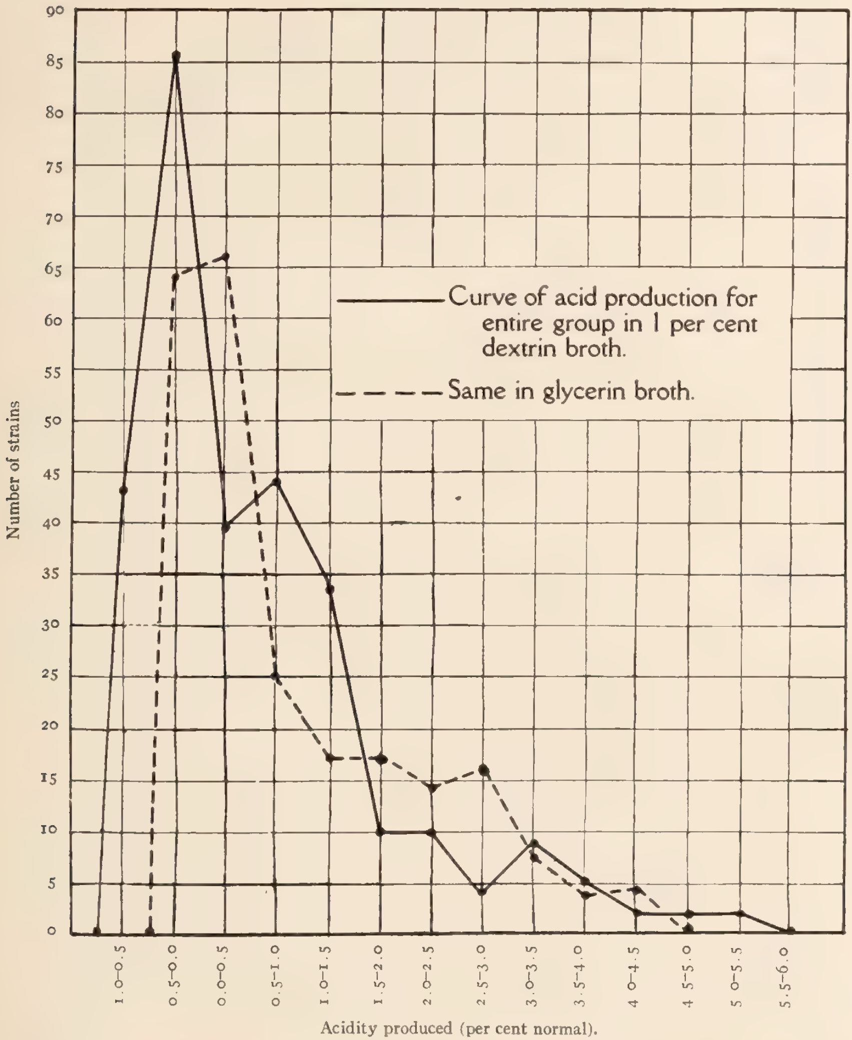

CHART 2.

No special stress can be laid on quantitative results in the fermentation of glycerin, because of the great variability of reaction to it. Table 3, however, does bring out two important points, i.e.,

that groups IV and VI do not acidify glycerin, while V has considerable fermentative powers.

TABLE 3.

CORRELATION BETWEEN MORPHOLOGICAL TYPES AND FERMENTATION OF GLYCERIN. ACID PRODUCED (PERCENTAGE NORMAL).

Types	Number of Cultures of Each Type					
	Alkaline −0.0	0.0–1.0	1.0–2.0	2.0–3.0	3.0–4.0	Above 4.0
I	19	36	20	19	4	0
II	2	16	9	8	[illegible]	0
III	4	6	2	[illegible]	0	0
IV	23	16	0	0	0	0
V	5	10	3	3	5	1
VI	15	8	0	0	0	0

Analysis of the results in maltose broth (Table 4) again brings out similarity between groups I and II and slight fermentative powers in III and VI. Thirty-nine, or 81 per cent of type IV are either alkaline or below 1 per cent acidity. Members of V, on the other hand, are strong acid-producers.

TABLE 4.

CORRELATION BETWEEN MORPHOLOGICAL TYPES AND FERMENTATION OF MALTOSE. ACID PRODUCED (PERCENTAGE NORMAL).

Types	Number of Cultures of Each Type					
	Alkaline −0.0	0.0–1.0	1.0–2.0	2.0–3.0	3.0–4.0	4.0–5.0
I	0	33	37	4	1	0
II	3	14	15	3	0	0
III	5	3	1	0	0	1
IV	27	12	2	5	1	1
V	2	5	3	3	6	9
VI	11	9	1	0	0	0

In saccharose broth (Table 5) groups I, II, and VI are gathered about the neutral point. Of groups III and V, half are below

TABLE 5.

CORRELATION BETWEEN MORPHOLOGICAL TYPES AND FERMENTATION OF SACCHAROSE. ACID PRODUCED.

Types	Number of Cultures of Each Type								
	Alkaline −0.0	0.0–1.0	1.0–2.0	2.0–3.0	3.0–4.0	4.0–5.0	5.0–6.0	6.0–7.0	Above 7.0
I	43	35	1	1	5	1	0	0	0
II	45	18	1	1	0	0	0	0	0
III	10	9	3	7	6	1	1	2	0
IV	[illegible]	3	4	9	23	8	9	5	3
V	19	7	6	2	3	2	1	0	0
VI	16	9	0	1	0	0	0	0	0

1 per cent acidity, the other half range to high acidities. Group IV is composed of strong acid-producers.

Dextrin (Table 6) is acted on to the same extent by types I and II, but is not decomposed by the last four.

TABLE 6.

Correlation between Morphological Types and Fermentation of Dextrin. Acid Produced (Percentage Normal).

Types	Number of Cultures of Each Type						
	Alkaline —0.0	0.0–1.0	1.0–2.0	2.0–3.0	3.0–4.0	4.0–5.0	5.0–6.0
I	16	32	30	11	8	1	0
II	5	25	10	4	3	0	1
III	6	16	4	0	1	1	0
IV	27	12	3	0	0	0	0
V	12	23	0	0	0	0	0
VI	16	5	0	0	0	0	0

Correlations between morphological types and virulence.—A study of the relationship between morphological types and virulence for guinea-pigs brings out the fact that of type I, 57 cultures or 61.3 per cent were virulent and 36 or 38.7 per cent non-virulent; while of type II, 17 cultures or 33.3 per cent were virulent, and 34 or 66.6 per cent non-virulent. Type III showed 18 non-virulent and only one virulent culture.

TABLE 7.

Correlation between Morphological Types and Virulence for Guinea-Pigs.

	Number of Cultures of Each Type					
	I	II	III	IV	V	VI
Virulent	57	17	1	0	0	0
Non-virulent	36	34	18	18 (tested)	10 (tested)	21 (tested)

Six cultures of class IV and three of V were tested for toxin production. Injections of the filtrate from two strains of class IV produced local induration in doses of 0.1 c.c., from another strain in a dose of 1.0 c.c. All of the animals recovered. Filtrates of the other six cultures were without effect in doses of 1 c.c.

Correlation between morphological types and chromogenesis.—The great majority (90 per cent each) of cultures of types I and II were gray. There were a few colorless, white, and yellow-white variants,

and a few (3 per cent each) showing a salmon-pink tinge. The proportion of the latter rose in type III to 23 per cent. In IV salmon-pink was the prevailing color (83 per cent), the remainder of the group being yellow, yellow-white, or white. Type V is represented in all classes except gray from colorless to yellow, but, as will be seen later, it is made up of two subdivisions, slowly and scantily growing strains, showing a colorless or white growth and differing slightly in fermentation reactions from the abundantly growing yellow and yellow-white cultures.

Most of the members of type VI gave a white or yellow-white growth.

TABLE 8.

CORRELATION BETWEEN MORPHOLOGICAL TYPES AND CHROMOGENESIS.

Type	Number of Cultures in Each Class					
	Colorless	White	Gray	Yellow-white	Yellow	Salmon-pink
I	3	1	101	3	0	4 (tinge)
II	1	0	46	3	0	2 (tinge)
III	1	0	16	3	0	6
IV	0	1	0	4	3	40
V	7	7	0	8	10	0
VI	1	15	0	8	0	2

Correlation between morphology and vigor of growth.—This is of limited value because, as has been said, a strain which grew poorly at first may later become accustomed to the medium, and flourish abundantly. The vigor of growth, therefore, has been estimated, not from the first culture alone, but also from successive replants.

Table 9, however, shows several important facts. The majority of cultures of type III grew heavily from the start. Strains of group IV all grew abundantly. In group V, two subdivisions are again apparent, as in Table 8, the scantily growing cultures being

TABLE 9.

CORRELATION BETWEEN MORPHOLOGY AND VIGOR OF GROWTH.
MORPHOLOGICAL TYPES.

Surface Growth	Numbers of Cultures of Each Type					
	I	II	III	IV	V	VI
Scanty	35	18	2	0	13	8
Medium	44	16	6	3	1	3
Heavy	33	18	17	45	18	15

colorless or white, and the heavily growing strains yellow or yellow-white.

The numbers of groups I, II, and VI in the different classes of growth are not especially significant.

From the preceding analysis, it is clear that there are two large subdivisions in the diphtheria group. The first is composed of slender, curved, granular, or segmented bacilli (types I and II) which vary greatly in morphology and develop characteristic involution forms. The members of this subgroup form acid in dextrose and maltose, and in about half the cases in glycerin and dextrin also, but they do not act on saccharose. The prevailing color of these cultures is gray. In man and in guinea-pigs many members of this group produce characteristic lesions due to a specific toxin.

The members of the second subdivision, which includes types IV, V, and VI, show a marked morphological resemblance to each other, and a lesser resemblance to members of the first subgroup. They are thicker and more regular than the latter and do not develop the same involution forms; they are non-virulent for guinea-pigs and do not form the toxin characteristic of the first group. The possibility of a pathogenic action for man will be touched on later. They show differences in fermentation reactions, agreeing only in their inability to acidify dextrin. Chromogenic cultures (yellow and salmon-pink) are frequent. All degrees of growth, from scanty to very heavy, are represented. The correlated differences in fermentation reactions, chromogenesis, and vigor of growth permit a further subdivision of this group into species.

There are also a few strains which, while coinciding in most properties with the first group, show features characteristic of the second. These cultures are represented by morphological type III.

This primary subdivision is a justification by the biometrical method of the current broad classification of the diphtheria group into the diphtheria bacillus and the "diphtheroid" bacilli.

Each of these groups will be considered in detail.

FIRST SUBGROUP, THE DIPHTHERIA BACILLUS (MORPHOLOGICAL TYPES I, II, AND III).

A glance at the table of relationship between morphology and the source of the culture, i.e., whether from a clinical case, a carrier,

or from an unusual situation or lesion, shows that, while types I and II were represented in approximately equal numbers among clinical cases and carriers, the bacilli from unusual situations were, with one exception, of type II.

TABLE 10.

CORRELATION BETWEEN MORPHOLOGY AND HABITAT OR LESION.

Type	Pharyngeal Diphtheria	Nasal Diphtheria	Carriers	Other Situations
I............	41	6	20	1-sinus of elbow joint
II............	37	2	18	2-eye 1-abscess of breast 1-sputum asthma 1-vagina 1-nose, with ozena bacillus
III............	5	0	16	0

Cultures from unusual situations include two from the eye, and one each from a sinus leading to the elbow joint, an abscess of the breast, the sputum from a case of asthma, the vagina, and the nose, associated with ozena bacillus. The majority of cultures of type III came from carriers, and only a small proportion from clinical cases (16 and 5, respectively).

In other words, diphtheria bacilli from parts of the body other than the throat and nose are more often of the segmented than of the granular type, and the small granular and solid forms are not often found in clinical diphtheria.

Correlations between sources of cultures and their virulence do not yield any new information. Of cultures taken from cases which the records sent to the Health Department showed to be cases of clinical diphtheria, 40 were virulent and 29 avirulent for guinea-pigs. Of cultures from carriers, mostly school-children, 13 were virulent and 37 avirulent. Cultures for release showed one virulent and three non-virulent. From cases of nasal diphtheria seven strains were virulent, and one non-virulent.

Five non-virulent strains were obtained from the nose, throat, and ears of scarlet fever patients. Two virulent cultures were obtained from the eye.

Cultures from unusual situations (abscess of the breast, sputum in a case of asthma, vagina) were non-virulent, with one exception—

a strain from a sinus following a fracture. The correlation of virulence with the three morphological types has already been devel-

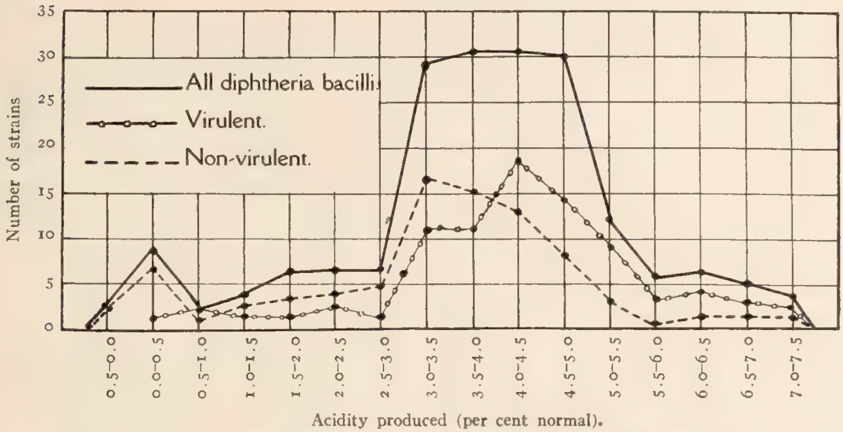

CHART 3.—Curves of acid production for types I, II, and III (diphtheria bacilli) in one per cent dextrose broth.

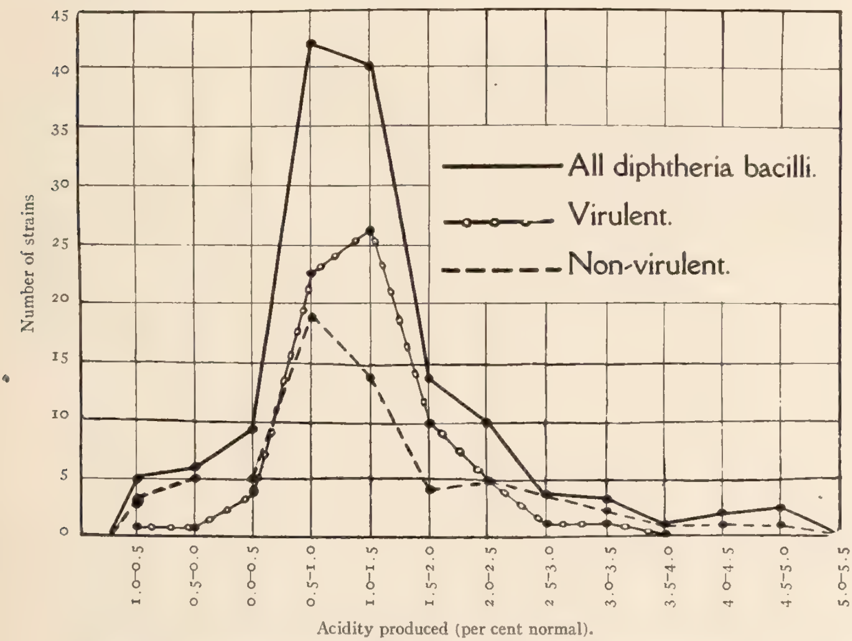

CHART 4.—Curves of acid production for types I, II, and III (diphtheria bacilli) in one per cent maltose broth.

oped. Of the granular cultures, the majority are virulent, while of the segmented two-thirds are non-virulent. The small granular type are almost all non-virulent.

The curves of acid formation for types I, II, and III (Charts 3 to 7) will be considered next. Those in maltose, saccharose, glycerin, and dextrin show only one mode. In dextrose broth, there

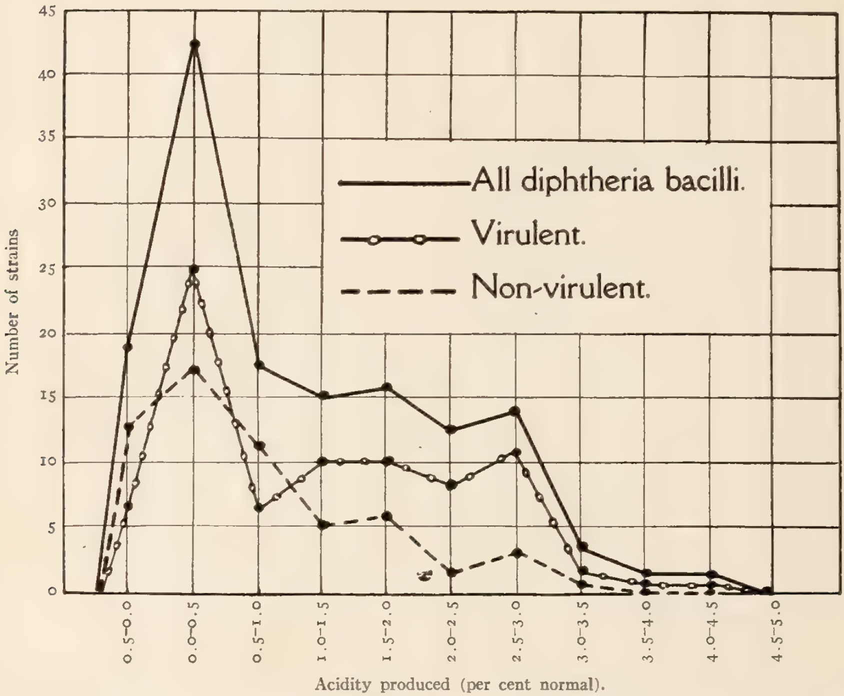

CHART 5.—Curves of acid production for types I, II, and III (diphtheria bacilli) in one per cent glycerin broth.

are two modes, a slight one at 0.0–0.5, and the chief one at 3.0–5.0 per cent. Plotting the curves for virulent and non-virulent diphtheria bacilli separately brings out slight differences between the two groups. In dextrose, maltose, and dextrin, the mode for the non-virulent series falls in each case at a lower degree of acidity than that for the virulent, while in saccharose most of the bacilli forming acid are non-virulent.

Analysis of the curve in dextrose broth shows that non-virulent

strains are chiefly responsible for the low acid group (nine non-virulent and one virulent). The mode for the high-acid group

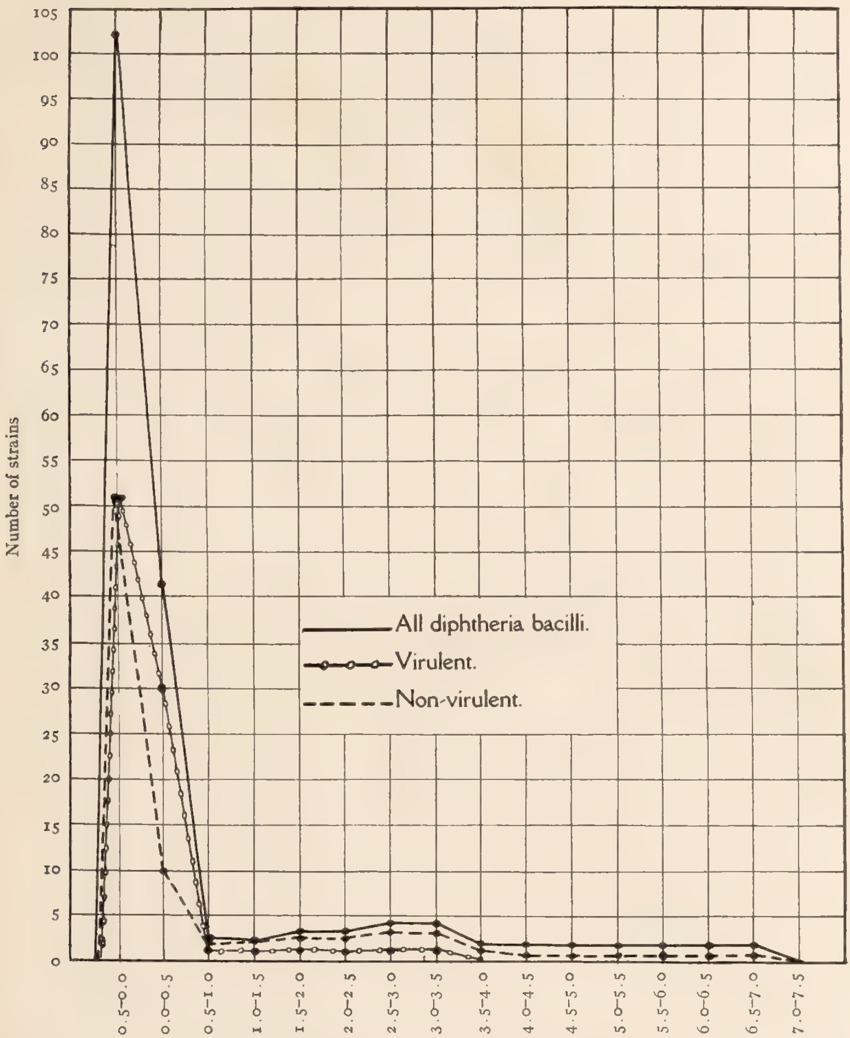

CHART 6.—Curves of acid production for types I, II, and III (diphtheria bacilli) in one per cent saccharose broth.

falls at 3.0–3.5 per cent for the non-virulent organisms; at 4.0–4.5 per cent for the virulent.

In maltose broth, the mode for the non-virulent strains is at 0.5–1.0, for the virulent at 1.0–1.5 per cent.

In dextrin broth, both virulent and non-virulent bacilli are represented to the same extent in the alkaline group, but the second mode for the non-virulent organisms is at 0.0–0.5 per cent; for the virulent at 1.0–1.5 per cent. Among the non-virulent there are no strains giving an acidity above 3.5 per cent.

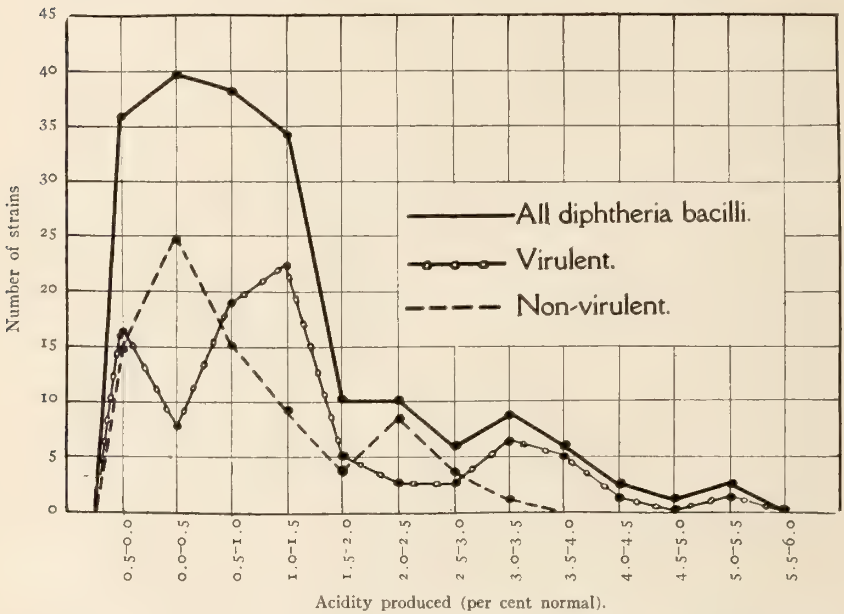

CHART 7.—Curve of acid production for types I, II, and III (diphtheria bacilli) in one per cent dextrin broth.

In glycerin broth, there is no difference between virulent and avirulent groups, the mode for both falling at 0.0–0.5 plus.

It has been a debated point whether or not differences, morphological or biochemical, could be demonstrated between virulent and avirulent diphtheria bacilli. These results show that when a long series is examined, distinct though small differences do appear in the groups as a whole, both in morphology and in fermentative powers. In the virulent group, granular bacilli predominate, forming 76 per cent; segmented organisms form 22 per cent, and small granular and solid types about one per cent. In the avirulent

group, granular and segmented forms are present in about equal proportions (the former 40 per cent, the latter 38 per cent), while 21 per cent of the group is made up of small granular and solid types. The virulent organisms have slightly greater action on dextrose, maltose, and dextrin. On the other hand, non-virulent organisms predominate among the saccharose fermenters.

It is apparent, however, that virulent and non-virulent forms are very closely related, and that the practice, common among medical workers, of limiting the term "true diphtheria bacillus" to the virulent forms, and using the expression "diphtheria-like" bacilli for the non-virulent, is in reality confusing and not justified biologically. In view of the ease with which virulence is known to be modified in other bacteria, it seems questionable whether it should be considered as having more than varietal significance.

The aberrant character of type III in relation to I and II and its similarity to type IV has been brought out. The resemblance is shown by its heavy growth, often with a pink tinge, its relatively slighter action on dextrose, maltose, and glycerin, on the one hand, and, on the other, its frequent fermentation of saccharose. These strains should perhaps be classed with diphtheroids rather than with diphtheria bacilli, but it is clear that no sharp line can be drawn biologically between these organisms and the typical diphtheria bacillus on the one side and group IV of the diphtheroids on the other. The typical forms of diphtheria and diphtheroid bacilli are of course widely separated, but there have been found in the course of this study a number of cultures which showed one or more of the properties considered characteristic of diphtheroids (e.g., chromogenesis, or fermentation of saccharose) while in other properties resembling the usual diphtheria bacillus. One of these strains produced sufficient toxin to kill a guinea-pig within two days when injected in a dose of 0.1 c.c.

SECOND SUBGROUP. THE SO-CALLED DIPHTHEROIDS AND HOFMANN'S BACILLI. (MORPHOLOGICAL TYPES IV, V, AND VI).

The members of this subgroup present certain general characteristics. Their morphology is distinctive. All that were collected during this work were gram-positive and non-motile. Neither the

gram-negative diphtheroid bacilli described by Hamilton[1] nor the motile forms described by DeWitt[2] have been observed in this study.

Neisser's granules were as a rule present in types IV and V, only five strains failing to produce them at the end of four days. In a number of cases, however, they developed late, being absent in 24-hour cultures, but numerous at the end of four days. Differences in the size and form of the granules were observed in the two types, as has been mentioned. No granules were found in type VI.

On serum, the majority of the strains produced heavy salmon-pink, yellow, or yellow-white growths. Colonies of the primary growth frequently were invisible for several days, but replants usually grew quickly and abundantly. Some strains, however, showed only a slow, scanty, colorless growth.

All the organisms were isolated from the human body, where these bacilli are not infrequently found in a variety of situations, both in normal and in pathological conditions. They probably would be found more often if routine cultures were examined again after the lapse of several days or a week.

The characteristics on which further subdivisions of this group are based are fermentation reactions, vigor of growth, and chromogenesis.

The curves of acid-formation in dextrose, maltose, and saccharose are alone significant, as dextrin is not acted upon by any of the organisms and glycerin only by a few of type V.

In dextrose broth (Chart 8), two groups are apparent, one non-acid, the other highly acid. Between these there are numerous intergrading forms. The first group comprises type VI, and a few strains mostly of type IV, the second, the majority of IV and V.

The curve in maltose broth (Chart 8) likewise shows two groups: an alkaline and faintly acid group, and an acid group, with its mode at 2.0–2.5 per cent. In the first and largest group are classes III and most of IV; in the second class V and a few of IV.

The curve in saccharose broth (Chart 8) has two sharply defined modes, one alkaline, the other at 3.0–3.5 per cent acid. Around the first mode are gathered types VI and V, while around the second is type IV.

[1] *Jour. Infect. Dis.*, 1907, 4, p. 326. [2] *Ibid.*, 1912, 10, p. 36.

A study of the relation of surface growth and chromogenesis to these curves helps to bring out the grouping. In dextrose broth 10, or 50 per cent, of the poorly growing strains are either alkaline or below 1 per cent acidity (Table 11). Of the heavily growing

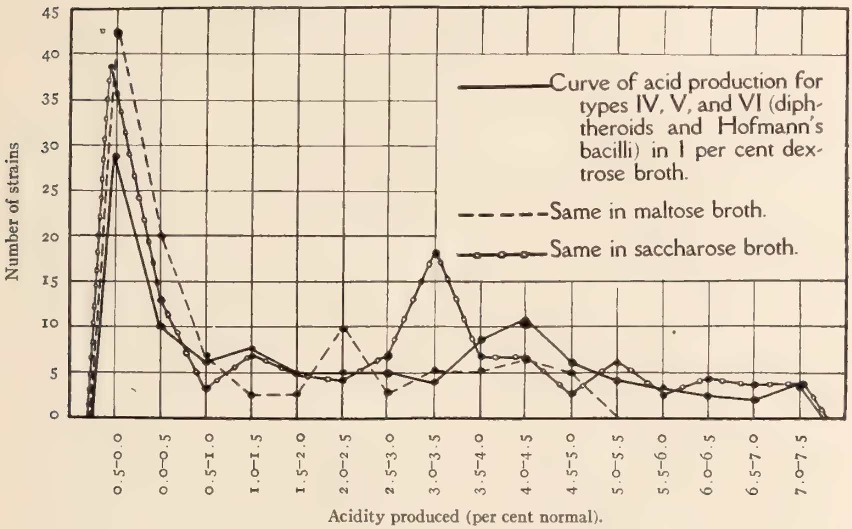

Chart 8.

cultures, 32, or 40 per cent, fall below 1 per cent and 29, or 36 per cent, above 3 per cent acidity. The majority of the low-acid, heavily growing cultures belong to type VI, which shows wide variation in

TABLE 11.

Correlation between Vigor of Growth and Fermentation of Dextrose. Acid Produced (Percentage Normal).

	Number of Cultures in Each Class					
	Alkaline − 0.0	0.0–1.0	1.0–2.0	2.0–3.0	3.0–4.0	Above 4.0
Scanty	9	1	5	2	2	1
Medium	2	0	1	0	2	3
Heavy	25	7	8	11	7	22

abundance of growth; the remainder belong to type IV. The heavily growing, high-acid group is composed of chromogenic cultures, chiefly yellow and salmon-pink, of types IV and V.

Correlation between degrees of surface growth and fermentation of maltose (Table 12) shows that 16, or 75 per cent, of the scantily growing forms are below 1 per cent acidity, and also 50, or 74 per cent, of the heavily growing strains. Here again, the scantily growing organisms belong to type VI, the heavily growing to types IV and VI. The acid-producers are of type V.

TABLE 12.

CORRELATION BETWEEN VIGOR OF GROWTH AND FERMENTATION OF MALTOSE. ACIDITY PRODUCED (PERCENTAGE NORMAL).

	Numbers of Cultures in Each Class					
	Alkaline −0.0	0.0–1.0	1.0–2.0	2.0–3.0	3.0–4.0	Above 4.0
Scanty	10	6	1	0	3	0
Medium	2	0	0	1	1	3
Heavy	46	4	3	6	4	4

The table of relationship between surface growth and fermentation of saccharose (Table 13) shows that 11 of the poorly growing strains are below 1 per cent, and only three above 2 per cent acidity. The heavily growing organisms are gathered at the two ends of the scale. The scantily growing non-acid strains are, as previously, of type VI, while the heavily growing non-acid group is composed of types V and VI. The highly acid group is formed by type IV.

TABLE 13.

CORRELATION BETWEEN VIGOR OF GROWTH AND FERMENTATION OF SACCHAROSE. ACID PRODUCED (PERCENTAGE NORMAL).

	Number of Cultures in Each Class					
	Alkaline −0.0	0.0–1.0	1.0–2.0	2.0–3.0	3.0–4.0	Above 4.0
Scanty	9	2	5	0	2	1
Medium	6	3	1	0	1	2
Heavy	27	5	5	11	18	22

Taking up chromogenesis more in detail, it is seen from Table 14, that nearly all of the colorless strains grew poorly, while the yellow-white, yellow, and salmon-pink cultures grew abundantly. The white strains were intermediate in position.

Correlations between chromogenesis and acid-production in dextrose, maltose, and saccharose are significant in grouping the organisms.

TABLE 14.

Correlation between Vigor of Growth and Chromogenesis.

	Number of Cultures in Each Class				
	Colorless	White	Yellow-white	Yellow	Salmon-pink
Scanty	13	5	1	0	0
Medium	1	3	2	2	0
Heavy	1	8	19	11	41

In dextrose broth (Table 15) 60 per cent of the colorless and 70 per cent of the white strains fall below 1 per cent acidity. These represent type VI. The yellow-white cultures show an even distribution from alkaline to high acidities. The non-acid yellow-white strains belong to type VI, while the acid-producers are allied in other characteristics to the next group, the yellow, all of which are above 1 per cent acidity. The salmon-pink cultures show a wide range of acidity, 30 per cent being below 1 per cent acidity, 24 per cent from 1 to 3 per cent, and 46 per cent above 3 per cent acidity.

TABLE 15.

Correlation between Chromogenesis and Fermentation of Dextrose.
Acid Produced (Percentage Normal).

	Number of Cultures in Each Class					
	Alkaline −0.0	0.0–1.0	1.0–2.0	2.0–3.0	3.0–4.0	Above 4.0
Colorless	7	2	3	2	1	0
White	9	1	1	0	2	1
Yellow-white	5	4	2	4	3	5
Yellow	0	0	1	1	1	7
Salmon-pink	6	7	7	4	6	15

In maltose broth (Table 16) the distribution of the colorless, white, and yellow-white strains is similar to that in dextrose broth, 83 per cent and 70 per cent of the colorless and white cultures respectively, giving less than 1 per cent acidity. Of the yellow strains five out of seven are decided acid producers. On the other hand, 86 per cent of the salmon-pink cultures fall below 1 per cent acidity.

In saccharose broth (Table 17) also, the majority of the colorless and white strains (60 per cent and 84 per cent, respectively) give less than 1 per cent acidity. Of the yellow cultures, 66 per cent

TABLE 16.

CORRELATION BETWEEN CHROMOGENESIS AND FERMENTATION OF MALTOSE.
ACID PRODUCED (PERCENTAGE NORMAL).

	Number of Cultures n Each Class					
	Alkaline −0.0	0.0–1.0	1.0–2.0	2.0–3.0	3.0–4.0	Above 4.0
Colorless	3	7	1	0	1	0
White	3	7	1	0	2	1
Yellow-white	2	4	1	3	1	4
Yellow	1	1	0	1	3	1
Salmon-pink	30	7	1	3	1	1

are alkaline, the remainder are above 2 per cent acidity. The salmon-pink cultures show here the reverse of their action in maltose broth, i.e., 70 per cent give more than 3 per cent acidity.

TABLE 17.

CORRELATION BETWEEN CHROMOGENESIS AND FERMENTATION OF SACCHAROSE.
ACID PRODUCED (PERCENTAGE NORMAL).

	Number of Cultures in Each Class					
	Alkaline −0.0	0.0–1.0	1.0–2.0	2.0–3.0	3.0–4.0	Above 4.0
Colorless	2	14	2	0	1	1
White	10	6	2	0	1	0
Yellow-white	8	3	4	3	5	2
Yellow	10	0	0	1	1	3
Salmon-pink	2	3	3	6	16	18

It is apparent from the preceding analysis, first, that the colorless and white cultures show two subdivisions, a small acid-producing group, having a meager growth on serum, and a more abundantly growing group, of morphological type VI, which does not act on dextrose, maltose, saccharose, or glycerin; second, that the salmon-pink strains all grow heavily, are of morphological type IV, and usually ferment dextrose and saccharose, but not maltose or glycerin; third, that the yellow cultures belong to type V, grow heavily, and act on dextrose, glycerin, and maltose, but not on saccharose.

Further study of the colorless and white cultures which produced acid shows that they all grew slowly and scantily, that they usually belonged to morphological type V, that they all fermented dextrose, 77 per cent fermented maltose, and 72 per cent saccharose.

From this mass of detail concerning the diphtheroids, four groups stand out plainly. These may be defined as follows:

Group A.—This is the largest numerically. The bacilli correspond to the "organism x" described by Hoag[1] from the Danvers State Hospital. It is a medium-sized bacillus, showing solid, barred, and wedge forms, with abundant but small and imperfect granules. On serum it produces a very heavy, confluent, glistening growth with a characteristic salmon-pink color. It ferments dextrose and saccharose, but not maltose or glycerin.

Group B.—The organisms of this group are usually larger than those of Group A, and thick forms with clear-cut bars predominate. Neisser's granules are very large and irregular. The growth on serum is heavy and varies in color from white to yellow. It is often noticeably dry and granular. Dextrose is always fermented, maltose and glycerin usually, but not saccharose.

Group C.—This, the smallest group, is differentiated primarily by its slow, scanty, colorless, or white growth. Morphologically, the organisms resemble Group B. They always acidify dextrose, and usually both maltose and saccharose.

Group D.—Composed of small, thick, straight bacilli, often barred and wedge-shaped, showing no granules. The growth on serum may be scanty or abundant and is white or yellow-white in color. They do not act upon dextrose, maltose, glycerin, or saccharose.

The members of Group B resemble the typical diphtheria bacillus in fermentation reactions more closely than members of the other groups, as both acidify dextrose and maltose but not saccharose. On the other hand, as has been said, certain intermediate strains of diphtheria bacilli are closely allied morphologically and culturally to Group A. Group D is obviously identical with Hofmann's bacillus. The xerosis bacillus corresponds in manner of growth and in fermentation reactions to members of Group C.

In regard to names for these species, it would seem that *B. hoagii* is appropriate for Group A, because the first extended description of it was given by Hoag, although bacilli apparently identical with it have been mentioned occasionally in the literature.

The salient characteristic of Group B, in addition to its fermentation reactions, is the yellow or yellow-white color of the cultures. The name *B. flavidus* might, therefore, be given to this species.

[1] *Boston Med. and Surg. Jour.*, 1907, 157, p. 10.

For Group C, it would appear best to retain the familiar name *B. xerosis*, limiting it, however, to bacilli showing the cultural and fermentative peculiarities of this group.

B. hofmanni is of course the rightful name for Group D.

The term "pseudo-diphtheria bacillus" is used with different meanings by different authors, some limiting it to Hofmann's bacillus and others applying it indiscriminately to any organisms resembling morphologically the diphtheria bacillus. The nomenclature here suggested eliminates this equivocal term.

Previous classifications of the diphtheroids have not been lacking, but they have been concerned chiefly with Hofmann's bacillus and the xerosis bacillus. Isolated strains of diphtheroids have been described and have been named from slight peculiarities in morphology, etc. Pyogenic forms have occasionally been mentioned. Classification has usually been based on morphology and the character of the growth on agar and gelatin, together with acid-production in glucose broth. Lehmann and Neumann[1] group the pseudo-diphtheria and diphtheroid bacilli with the diphtheria bacillus as corynebacteria, making the groups of *Coryn. diphtheriae; Coryn. pseudodiphtheriticus*, "which includes the luxuriantly, succulently, and rapidly growing non-virulent forms," and *Coryn. xerosis*, which designates the scantily and delicately growing varieties.

Hamilton[2] alone has differentiated groups based on fermentation reactions. She describes two groups of "pseudo-diphtheria bacilli" frequently found in suppurative processes, which are the same as groups A and B of the present study. She observed the same morphological and fermentative differences as are here described, but does not mention chromogenesis. Group B she considers very closely related to the true Klebs-Loeffler organism.

Although considerable experimental work has been done on *B. paralyticans*,[3] there appears to be in the literature no account of its fermentation reactions, except that it acidifies dextrose. During the present study a culture of this organism was obtained from Dr. Jessie W. Fisher of the Connecticut State Hospital, who

[1] *Bacteriology*, 1901, 2, p. 404.

[2] *Jour. Infect. Dis.*, 1907, 4, p. 326.

[3] Robertson, W. F., and McRae, G. D., *Rev. of Neurol. and Psych.*, 1903, 1, pp. 225, 305.

had received it from Dr. Ford-Robertson. It produced on serum, at the end of 24 hours, an abundant growth of isolated, moist, yellow-white colonies, the largest being 1 mm. in diameter. The growth was more translucent than that of *B. diphtheriae*. Morphologically, the organism was a large barred bacillus, often with tapering ends, and with numerous well-formed granules. The arrangement usually was parallel, and the bacillus was thicker and less curved than *B. diphtheriae*. At the end of four days the bacilli were fused into poorly staining masses, and no Klebs-Loeffler involution forms had developed. The bacillus formed much acid from dextrose, maltose, and saccharose, but did not affect glycerin or dextrin. It is apparent that this organism does not correspond in all its characteristics to any one of the groups. Its morphology, growth on serum, and action on maltose ally it to Group B, but contrary to the rule in this group it ferments saccharose.

Quite a number of the strains of diphtheroids investigated during this work were isolated from the lochia in puerperal infections, from the cervical discharge in pelvic inflammatory cases, and from vaginitis in children. Organisms which doubtless belong to this group have been found by Hallè[1] and by Foulerton and Bonney[2] in cases of puerperal fever, but it would seem that the frequency of these bacilli in the genital tract and their possible relation to gynecological infections has not been generally recognized. In our series, the bacilli were associated with streptococci in three cases, with staphylococci in ten, with gonococci in eight, while in eight cases no other bacteria were found in the smear or culture. In a series of control cultures from the cervix in pregnant and puerperal women with no pelvic lesions, the bacilli were not found. Their presence without other organisms suggests that in some instances they may be the cause of a mild inflammation in these situations. This point has not been investigated further. Diphtheroid bacilli in inflammatory processes usually have been regarded as saprophytes or harmless secondary invaders. Hamilton, however, believes that they play an important part in the causation of otitis media, especially the post-scarlatinal variety,

[1] *Ann. de Gynec. et Obst.*, 1899, 51, pp. 113, 195, 295

[2] *Trans., Path. Soc. London*, 1903, 54, p. 139.

because they were found in 72 per cent of 43 cases of acute scarlatinal otitis media, and in 20 per cent of these cases in pure culture; also because the opsonic index of the patients for these bacilli showed marked variations, and the injections of corresponding vaccines modified definitely the course of the infection.

CONCLUSIONS.

1. The diphtheria bacillus, Hofmann's bacillus, and the "diphtheroids" form one family.

2. The family is divided into two main subgroups, the first comprising the diphtheria bacillus, the second the "diphtheroids" and Hofmann's bacillus.

3. In the first group, it does not appear that any further differentiation can be made, except between the virulent forms and the non-virulent, which tend to have a heavier growth, more marked chromogenesis, more solid-staining forms, and lessened acid-production. The organisms of this group form a continuous series, and may well be retained under the species *B. diphtheriae*, and designated only as the virulent and non-virulent varieties.

4. The second subgroup can be further divided, on the basis of morphology, chromogenesis, fermentation reactions, and vigor of growth, into four species. These comprise the diphtheroids frequently found on the human body. In addition to the names *B. xerosis* and *B. hofmanni*, which are in common use, the terms *B. hoagii* and *B. flavidus* are suggested for these species.

Following is a table showing the characteristics of the five species in the diphtheria group.

TABLE 18.

	Morphology	Involution Forms	Neisser's Granules	Acid Production in					Toxin Production	Virulence	Chromogenesis	Vigor of Growth
				Dextrose	Maltose	Glycerin	Saccharose	Dextrin				
B. diphtheriae..	Slender curved, of variable length; granular or segmented	Characteristic	+	+	+	+	−	±	+	+	Gray	Medium or heavy
B. hoagii......	Small, thick, solid, segmented, or wedge-shaped	Bacilli fused into poorly staining masses	+ Small and indistinct	+	−	−	+	−	−	−	Salmon-pink	Very heavy
B. flavidus.....	Large, thick, with clear-cut segments	Usually no change but may be similar to *B. hoagii*	+ Large and irregular	+	+	+	−	−	−	−	White to yellow	Heavy
B. xerosis......	Resembles *B. flavidus*	No change	+ Usually large and irregular	+	+	±	+	−	−	−	Colorless or white	Scanty
B. hofmanni....	Small, thick, straight, solid, or wedge-shaped	No change	−	−	−	−	−	−	−	−	White or yellow-white	Scanty or heavy

ON THE TOXICITY OF BROTH, OF PNEUMOCOCCUS BROTH CULTURE FILTRATES, AND ON THE NATURE OF THE PROTEOLYTIC ENZYME OBTAINABLE FROM PNEUMOCOCCI.*

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THE TOXICITY OF BROTHS.

Although it is well known that peptone-broth and other mixtures of protein cleavage products are toxic when injected intravenously in animals, a comparative study of the toxicity of the various kinds of broth in common use, and of the increase in toxicity of broth which results from growth of pneumococci and other bacteria in relation to the amount of amino-nitrogen, would nevertheless seem to be desirable. The source, for example, of the toxic material which is formed when bacteria are grown in broth has not been established. Is it a part of the bacterial substance, an excretion of the bacterial cell, or an action of proteolytic and other enzymes on the protein constituents of the broth itself, or a combination of various factors? In the following pages are given the results of experiments bearing on these questions.

A study of the toxicity for guinea-pigs of intravenous injection of various samples of plain broth, containing the standard amount of Witte peptone and prepared with Liebig's beef extract, shows it to be relatively slight and quite uniform. Broth prepared from meat infusion instead of beef extract not only has a greater total toxicity, but some samples are much more toxic than others. The toxicity is greater when the beef is extracted at a relatively warm temperature. The toxicity of sugar-free meat broth, which is prepared exactly as the meat broth, but rendered free from sugar by fermentation by the colon bacillus, is the most toxic of all.

An average formol titration for 10 c.c. of each of these kinds of

* Received for publication July 18, 1912.

broth expressed in N/10 KOH is 1.2 c.c. to 1.6 c.c. for the plain broth, 1.8 c.c. to 2.3 c.c. for the meat broth, and 3.2 c.c. to 3.8 c.c. for the sugar-free meat broth. It is to be remembered that the amount of Witte peptone in all three varieties is the same. The source of the increased formol titration figure and the toxicity in sugar-free broth seems to be due to the action of the colon bacillus. Thus a sample of meat infusion prepared in the usual way titrated 1.7 c.c. before and 3.6 c.c. after the colon bacillus had grown in it for 24 hours at 37° C. The toxicity was correspondingly greater. The amount of fermentable sugar in such a mixture is shown to be slight by the small increase in acidity, while the protein splitting is correspondingly greater. The toxicity, in other words, is greater when protein degradation has taken place. The symptoms produced by the injection in proper amounts of the two kinds of broth are indistinguishable from those observed following immediate anaphylaxis, or following the injection of toxic pneumococcus, of peptone, or other bacterial extracts. Those following the injection of plain broth in large amounts are not so typical, bronchial spasm being less marked, symptoms of great weakness and paralysis more marked, and death not so sudden. From these facts it is evident that there is produced during the partial digestion of the meat, the result of bacterial action, a thermostable substance which is toxic for guinea-pigs much as are pneumococcus and other bacterial autolysates.

The effect of a solution of pure amino-acids, given to me by Dr. Woelfel, was tested by intravenous injection in guinea-pigs. They showed no noteworthy immediate symptoms and remained well even though very large doses were injected. Formol titration showed the presence of approximately 10 times the amount of amino-nitrogen present in the meat broth. The biuret reaction was negative. Hence the toxic substances in broth are formed during protein cleavage, as I have shown to be the case in pneumococcus autolysates, and would seem to be intermediate protein cleavage products. It is likely, however, that other enzymes than the proteolytic are present, and it might well be that these in some way have to do with the production of toxic material.

THE TOXICITY AND PROTEOLYTIC POWER OF FILTRATES OF PNEUMOCOCCUS BROTH CULTURES.

In order to test the proteolytic power of such filtrates, toluol was added to the clear fluid of broth cultures of four strains of virulent pneumococci; the filtrates were then placed at 37° C. Titration figures of one are sufficient. The acidity remained the same throughout the experiment which shows that the splitting was not the result of growth of pneumococci. At once the titration showed 4.5 c.c.; at the end of two weeks, 6.8 c.c., and at the end of two months 7.8 c.c. No increase occurred after two months even when new, heated, ascites-broth was added. The control mixture of heated ascites-meat-broth and toluol showed no splitting. To ascertain that the protein cleavage observed in filtrates of cultures of pneumococci in broth is not due to the increased acidity, control tests were made by rendering the reaction of the filtrate the same as the broth controls by means of sodium hydrate and by bringing broth to the same acidity as the filtrates by adding hydrochloric acid. In the former, splitting took place as rapidly as when the reaction was acid, while in the latter no splitting occurred. Experiments on the relative toxicity of culture fluids of pneumococci in meat broth and the corresponding broth show that there is a regular increase in the toxicity as pneumococci grow in broth and usually a corresponding increase in amino-nitrogen. The increased toxicity is not due to the increase in acidity which is produced by growth in media containing sugar.

In a previous paper I have stated that not all the toxic substances in infectious diseases may have their origin in the bacteria themselves, but that the mere growth of bacteria in the animal juices may also produce toxic material. The question whether the toxic substance comes from the pneumococci only or whether proteolytic action on the broth by the pneumococcus or its products may also produce toxic material was then considered.

Table 1 shows that during growth of pneumococci in broth there goes into solution a proteolytic ferment which, when added to meat broth in diminishing amounts, produces a proportionate amount of protein cleavage, and at the same time a corresponding increase in toxicity up to a certain point, after which the toxicity diminishes

even though formol titration increases. A closer study of the disappearance of toxicity after protein cleavage has reached a certain point, shows that disappearance occurs regularly when the cleared culture fluid is incubated and when it is added to the meat broth in varying amounts, but does not occur when pneumococci are allowed to remain or, if at all, until a much later period. The same is true when non-virulent cultures of pneumococci are incubated for a long time. In one sample the formol titration, the toxicity, and the viability of pneumococci were tested at intervals

TABLE 1.

PROTEOLYSIS IN MEAT BROTH DUE TO BROTH CULTURE FILTRATES OF VIRULENT PNEUMOCOCCI.

Mixtures	Formol Titration		Symptoms Following Intravenous Injection of 3.5 c.c. in (Duplicate) Guinea-Pigs
	Immediately	18 Days	
40 c.c. meat broth + 10 c.c. broth culture filtrate pneumococcus 622	3.0*	6.25	Definite but slight†
40 c.c. meat broth + 1 c.c. broth culture filtrate pneumococcus 622	2.7	4.2	Death in four minutes
40 c.c. meat broth + 10 c.c. NaCl solution	2.6	2.8	Slight

* The reaction of the mixtures showed no noteworthy change and hence is omitted in the table.

† The same quantity injected at the end of 10 days when formol titration figure was 4.8 produced death in three and four minutes. Cultures showed that the mixtures were sterile throughout the experiment.

for six months. The formol titration figure increased from 2.15 c.c. to 7.3 c.c. The toxicity remained high to the end of the experiment, and cultures showed the presence of living pneumococci in all the tests. This is interesting because filtrates of broth culture of non-virulent pneumococci contain little or no proteolytic enzyme, and it would therefore seem that here the proteolysis is due to bacterial growth.

THE PROTEOLYTIC POWER OF EXTRACTS IN NaCl SOLUTION OF PNEUMOCOCCI AND OTHER BACTERIA.

I have shown previously that when extracts and suspensions of virulent pneumococci and typhoid bacilli are placed at 37° C. there is a definite increase in amino-nitrogen. This does not occur in the case of extracts of non-virulent pneumococci, of streptococci, and of staphylococci. The increase in amino-nitrogen at the end

of 48 hours in meat broth cultures of these organisms is usually greatest in the former two, and hence it appears that in these a proteolytic enzyme goes into solution.

The extracts used in the experiments in Table 2 were prepared by suspending the growth of the various bacteria from 150 c.c. meat broth in 45 c.c. NaCl solution and placing them at 37° C. for 48 hours. Ether was added at once and then allowed to evaporate through the cotton plugs. The bacteria were removed by centrifugation and filtration through Berkefeld filters. The mixtures remained perfectly clear, and cultures on blood agar remained sterile throughout the experiment. The ascites-meat-broth was previously heated to 58° C. for 48 hours. The strain (602) of virulent pneumococcus had just been isolated from the blood of a fatal case of lobar pneumonia. The non-virulent strain (R51A) has been cultivated for 10 years. The streptococcus had been isolated from the throat in a case of scarlet fever three months previously. The staphylococcus came from a "malignant carbuncle" of the upper lip and was in the third generation. It is seen that the amino-nitrogen increased to a rather marked degree only in the broth to which was added the extract of the virulent pneumococcus. The control, the one containing extracts of the non-virulent pneumococcus and the streptococcus, remained the same while the mixture containing staphylococci showed a slight increase. The toxicity for normal guinea-pigs of the mixtures of ascites-meat-broth and NaCl solution, of the extracts of the non-virulent pneumococcus, of the streptococcus, and of the staphylococcus did not change. The mixture containing the extract of

TABLE 2.

Proteolytic Power of Extracts in NaCl Solution of Various Bacteria.

Mixtures 150 c.c. Heated Ascites-Meat-Broth to Which Is Added 30 c.c. NaCl Solution or Bacterial Extract as Follows:	Formol Titration				
	Immediately	48 Hours	4 Days	7 Days	18 Days
NaCl solution	2.45	2.5	...	...	2.7
Extract of highly virulent pneumococcus (602) in NaCl solution	2.5	3.3	3.2	3.9	4.7
Extract of non-virulent pneumococcus (R51A)	2.5	2.8	2.6	...	2.65
Extract of virulent streptococcus (595)	2.7	2.8	...	2.7	...
Extract of virulent staphylococcus	2.6	2.9	3.0	...	3.0

virulent pneumococci, while equally toxic in the beginning, was twice as toxic at the end of seven days. At the end of 18 days the toxicity, while greater than in the beginning, was distinctly less than at the end of seven days. The fact that no demonstrable difference in toxicity could be made out in those mixtures which show little or no difference in titration is good evidence that no splitting took place which might not be measurable by formol titration. It appears then that extracts of virulent pneumococci contain proteolytic enzymes which not only act on the pneumococcus proteins, as is the case in the autolysate, but which have the power to split foreign proteins such as are present in ascites-meat-broth. During this process there is produced highly toxic material which in its action on guinea-pigs is the same as that obtained from pneumococci, etc.

Quantitative studies show that while the filtered extracts and broth culture filtrates increase the toxicity of meat broth and heated ascites-meat-broth, as they cause protein splitting, the increase in toxicity in proportion to the protein splitting is less than when live pneumococci are allowed to grow in the corresponding broth. The total increase in toxicity is also greater in the latter. This is what one would expect because the pneumococci furnish a share of the material from which the toxic substance is made.

THE PROTEOLYTIC ENZYME OBTAINED FROM VIRULENT PNEUMOCOCCI.

This enzyme appears to have no power to split egg white and casein, but it does increase the amino-nitrogen somewhat when added to heated human and other sera, in which it also produces toxic substances. It is interesting to note that the proteolytic power of the extracts is greatest in those fluids which are particularly good culture media for virulent pneumococci.

When filtrates of pneumococcus in broth cultures are fresh and the proteolytic enzyme is still active, heating to 60° C. for one hour and boiling markedly reduce the toxicity. Heating the broth culture suspensions does not appreciably reduce the toxicity. This confirms my former statement that when clear toxic pneumococcus autolysates are heated the toxic property rapidly disappears,

whereas autolysates containing the pneumococci may remain toxic even after boiling for 10 minutes. This is likewise true when the clear filtrates are heated after the suspensions have been kept at 37° C. for a long time and the proteolytic enzyme is no longer active. Just as the proteolytic enzyme is more resistant to heat in the fluids of broth cultures, so it is more stable on standing. Thus in three of five extracts in NaCl solution, the digestive power over ascites broth was lost at the end of five months when kept in the ice-chest, while the other two still possessed it to a slight degree, whereas broth culture filtrates, when kept under the same condition, have been found to be quite active as long as 14 months after they were made. At room temperature and in the thermostat the ferment disappears more rapidly. In this connection it should be stated that the proteolytic power of extracts of pneumonic lungs has been found to behave similarly in these respects to the extracts in salt solution.

Ether and toluol reduce the action of the proteolytic enzyme obtainable from virulent pneumococci about one-half, while chloroform-water inhibits its action almost completely but does not destroy it. Formalin and bichlorid of mercury destroy its action in 1–1,000 solutions. The optimum reaction for the action of the proteolytic enzyme is between 0.5 per cent to 1.5 per cent acid to phenolphthalein. In weak alkaline or rather strong acid solutions it is entirely inactive.

In connection with the proteolytic power and the toxicity of pneumococcus extracts a study of their effect on potato oxydase has also been made. The bluing of guaiac was used as the indicator. There exists no relation between proteolytic power and the inhibitory action on potato oxydase. Study of a long series of extracts shows that usually the inhibitory power over potato oxydase is greatest at the time when the toxicity for guinea-pigs is greatest. To this there are exceptions for which there has thus far been found no adequate explanation. A further study of the action of these extracts on oxydizing processes is contemplated.

CONCLUSIONS.

Extracts of virulent pneumococci in NaCl solution and broth culture filtrates contain a proteolytic enzyme which hydrolyzes the

proteins contained in heated ascites-meat-broth, in meat broth, and to a lesser degree those contained in heated serum. During this digestion the toxicity of the broth is increased. The action of the toxic substance obtainable in this way is identical with the action of the toxic material obtainable from pneumococci and other bacteria and with the action of peptone. It does not attack egg white or pure casein.

The enzyme is more resistant to heat and long standing in the filtrates of broth cultures than in the extracts in NaCl solution. Heating to 60° C. for one hour reduces its action approximately one-half in the former and almost completely destroys it in the latter. Ether and toluol reduce the action one-half while chloroform-water inhibits it almost completely. Formalin and bichlorid of mercury destroy it promptly.

In the mixtures freed from pneumococci the increase of toxic substance or substances is in direct proportion to the increase of amino-nitrogen up to a certain point, after which toxicity grows less although protein cleavage continues. In the mixtures, however, from which the pneumococci are not removed, the diminution in toxicity is not observed.

The

Journal of Infectious Diseases

PUBLISHED BY THE MEMORIAL INSTITUTE FOR INFECTIOUS DISEASES

VOL. 11 *November 1912* NO. 3

EXPERIMENTAL STUDIES ON MILK.*†

WITH ESPECIAL REFERENCE TO THE UNIFORMITY OF DIFFERENT GRADES OF MILK, AND THE EFFECTS OF STORAGE UPON CERTIFIED, INSPECTED, AND PASTEURIZED MILKS.

BASED UPON DAILY OBSERVATIONS OF SAMPLES COVERING A PERIOD OF TEN MONTHS.

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INDEX AND OUTLINE.

*Received for publication June 27, 1912.

† Submitted as the thesis for the degree of Doctor of Public Health, Harvard Medical School, Boston.

I. CLASSIFICATION OF MILK.

The milk of our domestic animals, especially that of the cow, is one of the most important articles of food. All people are partially dependent on milk, and children, during the early period of their lives, are entirely dependent on it. It is an especially adapted food, for the carbohydrate is in solution ready for assimilation, the fat is already in emulsion, and the protein exists in such form that it is not coagulated by the heat at times necessary to preserve it and to render it innocuous. Milk readily deteriorates, can cause and carry disease, and is easily falsified. With the development of the commercial aspects of milk the dangers of harmful additions to and abstractions from milk have become as important as its necessity as a food. It is not to be wondered at that a satisfactory control of market milk should be demanded. In more recent years this control has not so much had as its object the prevention of frauds but rather has been designed to make available milk that is not harmful to the consumer. The milk

supplies of most large cities in the United States now consist of certified milk, inspected milk, raw market milk, and pasteurized milk.

Certified milk is milk obtained from healthy cows, free from tuberculosis as determined by the tuberculin test. The cows must be well fed and cared for, cleaned before milking, milked carefully and under sanitary conditions by clean milkers free from disease. This milk must be cooled and bottled soon after milking and must always be kept cold until it is delivered to the consumer. The bacterial count per cubic centimeter must be below 10,000. In some cities the percentages of butter fat and other chemical constituents are determined, while in other cities no particular attention is given to the chemical composition of certified milk. Some medical milk commissions demand a label stating the percentage of butter fat. Certified milk is generally, though not always, licensed by, and under control of, a local board appointed by a medical society.

Inspected milk is also obtained from healthy cows, free from tuberculosis. Milking, cooling, bottling, and handling must be done under sanitary conditions. Constant refrigeration too is required. The number of bacteria per cubic centimeter must not exceed 100,000.

Market milk is milk which in its raw state may or may not meet the requirements of certified and inspected milk.

Pasteurized milk is any raw milk heated for a time to a temperature above 139° F. and below the boiling point, after which it is rapidly cooled.

By far the largest amount of milk sold is market milk, either raw or pasteurized; certified milk is principally used by infants and invalids, while inspected milk is sold largely for family use. From October 1, 1910, to August 1, 1911, daily deliveries of 12 different supplies of milk from Boston were received at the laboratory. Of these 12 supplies, seven belonged to the certified class, two were inspected, and three were of milk pasteurized in bulk by the holding method. In addition during May, June, and July, 1911, inspected milk pasteurized in the sealed bottle at 150° F. for 30 minutes was received. All of these milks were examined when delivered and

after keeping them for varying periods of time at definite temperatures. All the tests available in the general laboratory were tried at these different examinations.

II. MILKS STUDIED.

CERTIFIED MILK.

No. 1. Milk from a mixed herd of about 40 cows, shipped to the city by train.

No. 7. Milk from a mixed herd of about 60 cows, hauled to the city by wagon. Only certified milk produced on this farm.

No. 8. Milk from a mixed herd of about 30 cows, shipped to the city by rail.

No. 9. Milk from a mixed herd of about 40 cows, shipped to the city by rail. Only certified milk produced on this farm.

No. 10. Milk from a mixed herd of about 40 cows, contained over $4\frac{1}{2}$ per cent of fat. Shipped to the city by rail. Only certified milk produced on this farm.

No. 11. Milk from a mixed herd of about 40 cows, shipped to the city by rail.

No. 14. Milk from a mixed herd of about 90 cows, hauled to the city by wagon. Only certified milk produced on this farm.

INSPECTED MILK.

No. 4. Supply made up of milk from a considerable number of farms, well inspected and controlled. Shipped to the city by rail.

No. 12. Milk from one farm, shipped to the city by rail.

PASTEURIZED MILK.

No. 2. Milk from tuberculin tested cows on several farms, pasteurized at 145° F. for 20 minutes by the holding device. Pasteurized from 12 to 24 hours before delivery.

No. 3. Market milk pasteurized at 145° F. for 20 minutes by the holding device. Pasteurized 12 to 24 hours before delivery.

No. 13. Market milk pasteurized at 145° F. for 20 minutes by the holding device. Pasteurized 12 to 24 hours before delivery.

No. $4\frac{1}{2}$. Inspected milk, pasteurized in the sealed bottle at 150° F. for 30 minutes under official supervision. Pasteurized 20 to 24 hours before delivery.

III. CONDITION IN WHICH MILK WAS DELIVERED.

All deliveries except of Nos. 4 and $4\frac{1}{2}$ were made in the morning, and usually were received in good condition. The drivers of the wagons were generally responsible, bright young men, and all but one were interested in delivering good milk. Milk was always received well refrigerated, but during the winter every supply except No. 4, which was delivered in the afternoon, was frozen at some time. This applied to certified as well as market milks. The greatest irregularity found was in the dating of certificates issued by the milk commissions. It not infrequently happened that certificates one to several months old were used and on investigation it was learned that the commissions had not supplied new certificates or had not prepared enough for the month. This condition did not occur as frequently after the summer of 1911 as before that time. Sour milk or curdled milk was never delivered. Pasteurized milk or any milk that has gone through a clarifier is generally somewhat more cream-colored than certified milk. Visible dirt was never found in the milk furnished, although at times the bottles were chipped, soiled from the ice, and not as carefully capped as they should be.

EXAMINATION OF MILK AS DELIVERED AND AFTER STORAGE.

Examination of milk is made to determine its food value and ingredients, the presence or absence of disease-producing elements, the presence or absence of the milk ferments, and to detect if there is any deterioration due to age, heat, or other processes. Methods of determining these facts differ and are numerous. In this investigation only those tests were made that deal with the hygienic or sanitary value of milk; furthermore only such tests were used as give results that may be easily and readily interpreted, for these, after all, are the ones that are of practical assistance, because a new supply of milk must be available daily. Officials in most cities acknowledge that there is no test immediately available by which

they can prevent the sale and consumption of milk that is not good —excepting, possibly, the temperature test, or the dirt test.

IV. SEDIMENTATION TESTS.

These tests are used principally to determine the presence of dirt and body cells, especially leukocytes. To detect these a number of methods have been devised. Dirt is frequently tested for by filtration through cotton, or by allowing large amounts of milk to stand in conical glasses. Usually garget is determined by straining through cloth or by smears made directly from milk[1] or from the sediment of centrifugalized milk. It was found that in the milks delivered, dirt was seldom demonstrable by filtration through cotton or by allowing it to stand, and that the making and examinations of smears requires much time and gives results which are not universally accepted.

The use of the Tromsdorff tube, which holds about 14 c.c. and has a small tube at the bottom, is satisfactory and gives reliable results. In these tubes 10 c.c. of milk is put and centrifugalized for 15 minutes. According to Auzinger[2] a sediment over 1.0 is unfavorable, and milk for infant feeding, from a mixed herd, should not be above one-fourth, and from a single cow not above one-half. Repeated microscopic examinations of the sediment in a Tromsdorff tube shows that a yellow sediment consists of cells, a white sediment is largely casein, and a gray sediment is due to dirt. According to Rühm leukocytes appear in milk at the beginning and end of lactation, due to accumulations of milk as the result of incomplete milking, from leukocytosis, as in fevers, etc., and in old milkers. Without going into the argument of leukocytes in milk, the results of the frequent determinations made with the Tromsdorff tube are given in Table 1.

From this table it is seen that relatively few samples were free from sediment. Market milk contains dirt much more frequently than certified or inspected milks. This is to be expected because market milk is made up of a mixture of milk from a large number of ordinary farms. Cells on the other hand occur more frequently

[1] Prescott and Breed, *Jour. Infect. Dis.*, 1910, 7, p. 632.

[2] *Ztschr. f. Fl. u. Milch-Hyg.*, 1910, 20, pp. 368, 400.

in certified and inspected milks than in the pasteurized market milk. This is undoubtedly due to the fact that the clarifier removes body cells more efficiently than finely divided dirt. Few of the

TABLE I.

SHOWING PERCENTAGE OF SAMPLES CONTAINING BODY CELLS AND DIRT, AND HIGHEST AND LOWEST TROMSDORFF READINGS.

Supply	No.	Body Cells			Dirt			No Sediment
		Percentage	Largest Amount	Smallest Amount	Percentage	Largest Amount	Smallest Amount	Percentage
Certified	1	43	$\frac{2}{3}$	0.0	57	Trace	0	0
	7	86	$1\frac{1}{4}$	0.0	14	Trace	0	0
	8	62	$\frac{1}{2}$	0.0	26	$\frac{1}{4}$	0	12
	9	50	$\frac{1}{4}$	0.0	50	$\frac{1}{8}$	0	0
	10	62	$\frac{1}{2}$	0 0	38	$\frac{1}{8}$	0.0	0
	11	100	$\frac{3}{8}$	Trace	0	0	...	0
	14	75	$\frac{1}{2}$	0	25	$\frac{1}{4}$	0.0	0
	Average	68	$1\frac{1}{4}$	0	30	$\frac{1}{4}$	0.0	2
Insp.	4	80	$\frac{3}{4}$	0	20	Trace	0.0	0
	12	75	$\frac{3}{8}$	0.0	12.2	$\frac{1}{8}$	0.0	12.5
	Average	77	$\frac{3}{8}$	0.0	16	$\frac{1}{8}$	0.0	7.5
Pasteurized	2	55.5	$\frac{1}{4}$	0.0	44.5	$\frac{1}{4}$	0.0	0.0
	3	62.5	$\frac{1}{1}$	0.0	27.5	$\frac{1}{4}$	0.0	.0
	13	28.5	$\frac{1}{8}$	0.0	71.5	$\frac{1}{8}$	0.0	0.0
	$4\frac{1}{2}$	78	$\frac{3}{8}$	0	22	$\frac{1}{8}$	0.0	0.0
	Average, 2, 3, 13	50	$\frac{1}{3}$	0.0	50	$\frac{1}{4}$	0.0	0.0

samples, however, showed sediment to the mark $\frac{1}{2}$, a large number only containing a trace. In only one sample was the sediment sufficient to exceed the mark 1.0, and this was due to body cells as confirmed by examination of a suitably stained slide. All of our better classes of milk contain little sediment.

V. BACTERIOLOGICAL EXAMINATIONS.

1. METHODS AND TECHNIC.

The bacteria in milk are of importance both from health and from commercial standpoints. As far as health is concerned it is especially important to know whether milk contains pathogenic bacteria. To determine this, however, requires time, labor, and expense, so that it is not feasible to examine all milk for specific microorganisms. Of the available routine examinations, the determination of the number of bacteria per cubic centimeter gives the most valuable information, because infection of the udder, dirty methods, poor refrigeration, and age all lead to higher bacterial

counts. In addition, however, to determining the bacterial count it is of importance to know the action the bacteria will have on the milk as well as the effect storage at certain temperatures will have on these microorganisms. For this reason determinations of the total number and of fermenting and peptonizing organisms in one cubic centimeter, and of the presence of organisms liquefying gelatin, producing indol and hydrogen sulfid were made on the different milks as received, and after varying periods of storage at definite temperatures. The number of bacteria per cubic centimeter was determined by the standard method as outlined by a committee of the American Public Health Association[1] in 1908. The number of organisms fermenting lactose was determined by adding 1 per cent of lactose to neutral litmus agar. To determine the number of bacteria breaking up the protein of milk, one cubic centimeter of sterile skimmed milk was put into the petri dish, after which the proper dilution of milk was added, and finally molten sugar-free agar was poured in. After 48 hours of incubation, a dilute solution of acetic acid was run over the plate, then the number of proteolytic organisms producing colonies surrounded by a clear zone was determined. This is essentially the method of Hastings.[2]

The amount of gas and the gas formula were determined in lactose broth in Smith fermentation tubes, according to the usual method. The fermentation tubes were inoculated with 1 c.c. of milk and incubated at 37° C. for 48 hours.

Liquefaction of gelatin was determined in sugar-free gelatin. One cubic centimeter of milk was put on the hardened gelatin, the height of gelatin in the tube being marked. After this a straight stab was made through the medium with the platinum needle. The stab was made so as to insure inoculation because the cream on rising carries with it so many bacteria that all the liquefiers may be carried away from the surface of the gelatin. These tubes were incubated at room temperature for 10 days.

Hydrogen sulfid and indol production were tested by inoculation of Dunham's solution with 0.1 c.c. of milk in a test tube containing a strip of filter paper saturated in lead acetate held by the cotton plug. The tubes were incubated for 10 days at 37° C. when

[1] *Am. Jour. Pub., Hyg.*, 1908, 18, p. 425.

[2] *Centralbl. f. Bakt. u. Parasit.*, 1902, 10 ,p. 384, and 1904, 12, p. 590.

the readings for hydrogen sulfid were made. After this indol was tested for in the regular way.

2. BACTERIAL COUNTS.

The total number of peptonizing and fermenting bacteria per cubic centimeter was determined at frequent intervals, and it seems sufficient to give the average and highest counts obtained.

BACTERIAL COUNTS OF MILKS AS RECEIVED.

The following table shows the results obtained in the examinations of certified, inspected, and pasteurized milk as it is delivered.

TABLE 2.

SHOWING AVERAGE, HIGHEST, AND LOWEST BACTERIAL COUNTS, AND MONTHS IN WHICH HIGHEST AND LOWEST COUNTS OCCURRED.

CERTIFIED MILK.

Number	Average Count	Highest Count	Lowest Count	Percentage over 10,000	Percentage 5,000–10,000	Percentage 1,000–5,000	Percentage less than 1,000	Time of Highest	Time of Lowest
1	3,756	10,500	800	6	35	47	12	January	November
7	6,650	27,000	1,800	31	38	31	..	November, 1910	August, 1911
8	8,935	24,000	1,400	36	21	43	..	November	May, June
9	20,650	51,000	3,500	75	..	25	..	November	February, June
10	13,900	52,000	900	56	18	18	8	November	May
11	3,543	14,000	600	7	15	57	21	March	June
14	3,470	7,100	800	0	9	73	18	November	August

INSPECTED MILK.

Number	Average Count	Highest Count	Lowest Count	Percentage 50,000–100,000	Percentage 10,000–50,000	Percentage 5,000–10,000	Percentage 1,000–5,000	Percentage less than 1,000	Time of Highest	Time of Lowest
4...	33,610	52,000	12,500	8	92	..	..	....	November	March
12...	11,545	99,000	1,300	21	28	23	28	....	June	March

PASTEURIZED MARKET MILK.

Number	Average Count	Highest Count	Lowest Count	Percentage over 1,000,000	Percentage 500,000–1,000,000	Percentage 50,000–500,000	Percentage 10,000–50,000	Percentage 5,000–10,000	Percentage 1,000–5,000	Percentage below 1,000	Time of Highest	Time of Lowest
2...	310,250	840,000	6,000	..	40	27	20	13	..	..	August	November
3...	285,875	1,640,000	16,000	..	53	31	16	..	..	..	February	November
13...	916,333	1,560,000	4,000	..	31	46	8	15	..	..	March	December
4½..	4,020	9,700	200	..	..	..	0	30	40	30	July	May

From Table 2 it will be seen that while the average bacterial count for certified milks was below the 10,000 maximum count allowed, still the producers of certified milks, numbers 7, 8, 9, and 10, had considerable difficulty in keeping below the maximum. When, however, the producer has properly adjusted conditions, he can produce milk of low count rather consistently as is the case with milks 1, 11, and 14. Of the inspected milks, 4, which is made up of milk from a number of farms, while never containing less than 12,500 bacteria per cubic centimeter, never contained above 50,000 and gave an average of 33,610. Inspected milk 12 had a bacterial content as low as 1,300, but also as high as 99,000, showing that at times there was a break in technic. Market milks 2, 3, and 13 contained rather large numbers of bacteria per cubic centimeter, for pasteurized milk. That pasteurization or storage after pasteurization must have been faulty is shown by the low counts observed at times.

In comparison with 2, 3, and 13, supply $4\frac{1}{2}$, which was heated in the sealed bottle to 150° F. for 30 minutes, never contained more than 10,000 bacteria per cubic centimeter, and went as low as 200 per cubic centimeter.

It is evident from these examinations and from observation at the place of production and distribution, that generally it is comparatively easy to produce milk of low bacterial count but that the greatest care must constantly be exercised if occasional high counts are to be avoided.

The time of the year when the higher counts occur varies. The better milks generally have highest counts in the fall and winter. Especially is this true of certified milks, while the general market milk is likely to show the highest counts in the spring and summer.

Investigation of the causes of the higher counts in certified milks during the fall seems to indicate that the necessity for carrying milk to the distant milk-house through the cold air makes the milkers loath to wash their hands before milking each cow, and that winter weather generally interferes with the rigid technic of certified farms. The higher counts in market milk during the summer are probably due to more pressing work on the farm, dust and multiplication of bacteria occurring as a result of poor refrigeration,

especially in the spring when winter methods are no longer efficient and summer refrigeration has not yet been well started.

BACTERIAL COUNTS AFTER STORAGE FOR VARYING PERIODS OF TIME AT DEFINITE TEMPERATURES.

Milk, when it is offered for sale, has been exposed to storage at various degrees of temperature. While certified and inspected milks are usually kept cold from the time of production to the time of sale, ordinary market milk, which forms by far the largest part of all milk consumed, frequently is not kept cold at the farm and during transportation.

With the institution of pasteurization, refrigeration before pasteurization is no longer deemed essential. In our cities very little milk is delivered to the consumer within 24 hours after production, a large portion of the pasteurized market milk is 48 hours old, and most milk is not consumed until it is 48 hours to 72 hours old. With the institution of efficient refrigeration some milk is stored for 10 to 30 days. It is thus seen that milk is held for a time at various temperatures, and to determine the bacterial multiplication the various milks studied were held at 37° C., room temperature, the ordinary ice-box temperature (45 to 55° F.), and at a temperature just above freezing. It was found that milk of all grades after storage at 37° C. and at the room temperature showed most variable rates of bacterial increase. Furthermore, the number of bacteria per cubic centimeter varied a great deal when coagulation occurred. After 12 hours of incubation at 37° C., certified, inspected, and pasteurized milks contained from 100 to 20,000 times the original number of bacteria, while at room temperature the rate of multiplication varied from 100 to 30,000 times the original number in 24 hours. At the temperature of the ice-box the number of bacteria for the first 24 hours varied from 20 to 200 times the original number, after 72 hours from 20 to 500 times the original number. When these milks were stored at a temperature just above freezing, little or no multiplication was observed during the first seven days, pasteurized and inspected milks showed more increase than certified milk, but after the 10th day the bacteria generally multiplied very rapidly in all milks. Realizing that in practice milk that is stored is generally not mixed until it is to be used, that the cream

on rising carries with it most of the bacteria, that when milk and cream have separated and are then mixed not nearly as large a proportion of the bacteria rise to the top, and that the rate of multiplication in cream and skimmed milk may be different, counts were made of milks that had been mixed several times as well as of milks that had not been mixed. The increase in bacteria at 1.0° C. is shown in the following table:

TABLE 3.

SHOWING AVERAGE INCREASE IN BACTERIA WHEN MILK IS STORED AT A TEMPERATURE JUST ABOVE FREEZING. FIGURES REPRESENT MULTIPLE OF ORIGINAL NUMBER PRESENT.

	7 Days' Storage	10 Days' Storage	15 Days' Storage		25 Days' Storage	
	Mixed 2 Times	Mixed 2 Times	Mixed 3 Times	Mixed Only after 15 Days	Mixed 4 Times	Mixed Only at End of 25 Days
Certified	35	640	4,250	1,800*	12,680	8,680*
Inspected	240	3,000	5,800	1,300	52,000	1,560
2, 3, and 13	344	4,450	114,20	420	72,420	920

* Multiple determination based on average number of bacteria per c.c., as shown in Table 2.

BACTERIA IN CREAM AND SKIMMED MILK.

When cream is allowed to rise on milk it carries with it a considerable portion of the bacteria present in milk. Knox and Schorer[1] found that after milk has remained in a gravity separator packed in ice over night nearly all the bacteria are to be found in the cream layer; Anderson[2] states that his studies show that top milk, such as is advised for use in preparing formulae for infant feeding, contains from 10 to 500 times as many bacteria per cubic centimeter as the mixed milk, from which the cream is obtained. In 26 samples of milk Anderson found that the gravity cream contained about four times as many bacteria as the sediment layer and about one-third as many as the whole milk. This, however, varies with the quality and age of the milk. Milk of high bacterial count well refrigerated while the cream is separating yields cream containing several thousand times as many bacteria as the skimmed milk, while the cream rising on milk of low bacterial content may contain only 10 to 15 times as many as the skimmed milk. The

[1] *Arch. Ped.*, 1907, 24, p. 516.

[2] *Jour. Infect. Dis.*, 1909, 6, p. 392; *Bull. Hyg. Lab.*, 1909, 56, p. 739.

same milk separated into cream and skimmed milk by gravity separation, as well as by centrifugalization in the test tube, yields cream which contains a relatively larger portion of the bacteria than does cream separated by the commercial cream separator. This is shown in the following test: the number of bacteria per cubic centimeter in the whole milk was 9,800; the gravity cream which rose in 12 hours contained 35,000 and the skimmed milk 610; cream on centrifugalization in the test tube contained 22,000, the skimmed milk 1,550; 16 per cent separator cream contained 15,500 and the skimmed milk 5,200.

To determine whether bacterial multiplication is more rapid in the cream or skimmed milk, resulting from gravity separation, whole milk was stored at 1.0° C. for 14 days and 25 days. At the time of examination the milk bottle was removed from the refrigerator and 1 c.c. of cream taken out and plated; after this a small amount of the skimmed milk was taken from the bottle by means of a sterile siphon and from this plates were made, and finally all of the remaining milk was well mixed by shaking and rolling the bottle, and from this mixed milk plates were made. There was considerable variation in the number of bacteria in the different portions after long storage. The average of these results is given in the following table:

TABLE 4.

SHOWING AVERAGE RELATIONS OF NUMBER OF BACTERIA AFTER STORAGE AT 1° C., IN CREAM AND MILK COMPARED TO THE MIXED MILK.

	STORAGE FOR 14 DAYS			STORAGE FOR 25 DAYS		
	Cream to Whole Milk	Skim Milk to Whole Milk	Cream to Skim Milk	Cream to Whole Milk	Skim Milk to Whole Milk	Cream to Skim Milk
Certified	80:1	1:1200	96,000:1	460:1	1:190	87,400:1
Market pasteurized	250:1	1:270	67,500:1	39:1	1:4	156:1

Not enough examinations of inspected milk were made to warrant a fair average for comparison.

No definite conclusions on rapidity of bacterial growth in cream and skim milk can be made from these observations, as not enough examinations were made on the fresh cream and skimmed milk to offer figures for comparison. However, it will be seen that the

bacterial content after long storage was more nearly the same in the cream and skimmed milk of pasteurized market milk than in that of certified milk. Whether this is due to pasteurization or mechanical mixing as occurs in clarifiers and heaters has not been determined.

3. BACTERIA PRODUCING PEPTONIZATION AND FERMENTATION.

Many kinds of bacteria occur in milk but among these the acid-forming and -digesting species abound. The flora in milk is largely dependent on environmental conditions such as temperature, moisture, amount and character of food, the relations between the microorganisms present, and relative number of one species to the numbers of other species. Under ordinary circumstances the acid-producing organisms play the principal rôle, but under a number of conditions the putrefactive bacteria may increase sufficiently to prove their characteristic putrefactive products.

Acid formation in milk results from the decomposition of the lactose, and if it is removed as has been done by a number of investigators, the general results of Babcock, Russell, Vivian, and Hastings[1] are obtained. Under these conditions more liquefying organisms are present and putrefaction changes occur. The putrefactive organisms grow better at low temperatures than do the acid-forming ones; therefore at low temperature of storage the acid-forming organisms are held in check. Ravenel, Hastings, and Hammer[2] have found that at 0° C. there is an increase in bacteria as well as an increase in the percentage of soluble nitrogen.

In pasteurization it is generally claimed that the acid-producing organisms are destroyed, while the spore-bearing, putrefactive types survive. Ravenel and Hastings[3] believe that the practical effects of cold storage are identical with pasteurization in this respect. Ayers and Johnson[4] have shown that acid-producing organisms are abundant in milk after commercial pasteurization. Most of the conclusions in regard to pasteurization are based on pasteurization at high temperatures and on laboratory tests made in a test tube or flask. Such pasteurization, however, does not

[1] *Wis. Agric. Exp. Sta.*, 1901, *18th Ann. Rept.*, p. 157.

[2] *Jour. Infect. Dis.*, 1910, 7, p. 38.

[3] *Ibid.*

[4] *Pub. Bureau of An. Ind.*, 1910, Bull. 126.

give the same results as commercial pasteurization at 140 to 145° F. in the generally used system of holding tanks, followed by cooling and bottling by means of machinery.

The predominance of acid-forming bacteria in market milk is probably due to the disproportion in numbers at the time of its production. The acid-producing bacteria in milk come largely from manure, while putrefactive types are introduced from the air, utensils, etc. With the improvement of technic in the production and distribution of milk, contamination has been reduced. This is especially true in regard to certified milk. In this grade of milk there is very little fecal contamination, so that the acid-forming organisms are largely eliminated. Protein-digesting bacteria, on the other hand, are not as efficiently eliminated because many of the groups of bacteria are not destroyed by the live steam resorted to for sterilization of pails, cans, bottles, and other apparatus necessary for cooling and bottling milk. For these reasons, even though certified milk contains fewer bacteria per cubic centimeter, the proportion of putrefying organisms to acid-forming species is higher in certified milk than in the common milks.

The importance of acid-forming and protein-digesting bacteria in milk being established, determinations of their presence and their

TABLE 5.

SHOWING PERCENTAGES OF ACID-FORMING AND PROTEIN-DIGESTING BACTERIA IN MILKS AS DELIVERED.

Supply	No.	Percentage of Lactose Fermenters			Percentage of Peptonizing Species		
		Average	Highest	Lowest	Average	Highest	Lowest
Certified	1	26.	70	0	40.8	60	15
	7	33.7	66	8	31.2	50	12
	8	58.0	80	30	16.0	20	8
	9	37.2	70	5	11.5	30	0
	10	31.4	50	14	15.8	40	0
	11	39.0	85	14	38.1	90	16
	14	33.2	70	10	43.2	80	70
Insp.	4	20.1	48	0	16.3	28	1
	12	45.	80	40	18.0	50	5
Pasteurized	2	34.1	80	0	13.7	60	0
	3	29.3	70	8	42.3	50	20
	13	38.3	50	20	38.0	80	15
	4½	29.6	70	0	18.6	50	0

relative proportions were made when the milks were delivered and after storage at different temperatures for varying periods of time.

To do this, lactose litmus agar and skimmed milk agar plates, sugar-free gelatin, sugar-free broth, and lactose litmus fermentation tubes were inoculated as has already been described (Table 5).

TABLE 6*a*.

SHOWING PERCENTAGE OF ACID-FORMING BACTERIA IN THE DIFFERENT GRADES OF MILK AS DELIVERED AND AFTER STORAGE.

	Milks as Received			37° C.		Room Temp.			Ice-Box Temp.			1° C.			
	Average	Highest	Lowest	12 Hrs.	Coagulation	24 Hrs.	48 Hrs.	Coagulation	24 Hrs.	72 Hrs.	Coagulation	7 Days	15 Days	25 Days	Physically Changed
Certified ...	35.6	80	0	26.5	47 0	28	27.0	46.5	29	33	47.4	13	33	30.0	24.5
Inspected ..	32.5	80	0	24.0	48.2	10	10.0	14.0	25	25	27.5	13	40	21.0	27.0
Pasteurized, 2, 3, 13 ..	33.2	80	0	10.0	38.0	20	8.5	14.0	21	15	37.0	23	30	48.2	28.0
4½	29.6	70	0	20.0	31.0	18	....	10.0	6	7	10.0	50	42	25.0	....

TABLE 6*b*.

SHOWING PERCENTAGE OF PROTEIN-DIGESTING BACTERIA IN THE DIFFERENT GRADES OF MILK AS DELIVERED AND AFTER STORAGE.

	Milks as Received			37° C.		Room Temp.			Ice-Box Temp.			1° C.			
	Average	Highest	Lowest	12 Hrs.	Coagulation	24 Hrs.	48 Hrs.	Coagulation	24 Hrs.	72 Hrs.	Coagulation	7 Days	15 Days	25 Days	Physically Changed
Certified....	29.6	90	0	17.5	22.5	11	12.1	18	21	25	12.5	22.3	24.1	32.2	40
Inspected...	16.9	50	1	....	....	10	....	..	8	5	10.0	11.0	17.5	12.0	50
Pasteurized, 2, 3, 13...	32.0	80	0	....	....	18	7.0	..	21	15	7.5	22.5	40.0	30.0	47
4½	18.6	50	0	....	....	..	....	..	3	..	....	16.0	30.0	....	..

From Tables 6*a* and 6*b* it will be seen that of the bacteria in good milks at the time of delivery about 30 per cent are fermenters, that the peptonizers constitute 20 per cent of the total number in inspected milks and 30 per cent in certified and pasteurized milks. At the higher temperatures the percentage of fermenting organisms increases more rapidly and markedly in certified milk than in inspected pasteurized milks, but at the lower temperatures the percentage of acid-producing organisms is higher in pasteurized milk than in certified milk.

The protein-digesting bacteria increase in proportion only at very low temperatures, the increase being about the same in all grades of milk.

One series of determinations was made on the percentages of acid-forming and protein-digesting types in the gravity cream and skimmed milk stored for 25 days at 1° C. The results are shown in the following table:

TABLE 6c.

SHOWING PERCENTAGE OF ACID-FORMING AND PROTEIN-DIGESTING BACTERIA IN CREAM AND SKIMMED MILK WHEN MILK WAS STORED WITHOUT MIXING FOR 25 DAYS AT A TEMPERATURE JUST ABOVE FREEZING.

	Whole Milk		Cream		Skimmed Milk	
	Fermenters	Peptonizers	Fermenters	Peptonizers	Fermenters	Peptonizers
	Percentage	Percentage	Percentage	Percentage	Percentage	Percentage
Certified	43	25	30	31	61	20
Inspected	50	25	15	30	40	10
Pasteurized, 2, 3, 13	35	17	33	43	30	3

While not many tests were made, only one quart of each supply, the results found are suggestive. Apparently the skimmed milk contains the larger percentage of acid-forming types and the cream the larger proportion of the protein-digesting bacteria. This may account for the early appearance of peptonization immediately under the cream line.

FERMENTATION OF LACTOSE IN FERMENTATION TUBES.

While by means of lactose litmus agar plates the percentage of acid-forming organisms can be obtained, it must be realized that to make plates for numerical determinations very small amounts of milk must at times be used. In milk containing only a few fermenting organisms these organisms may be lost because of dilution. To overcome this difficulty lactose litmus fermentation tubes were inoculated with 1 c.c. of milk. The results are shown in the following table:

TABLE 7.

SHOWING PERCENTAGE OF SAMPLES 1 C.C. OF WHICH CONTAINED AT THE TIME OF DELIVERY ORGANISMS PRODUCING GAS IN LACTOSE LITMUS FERMENTATION TUBES.

	Certified							Inspected		Pasteurized				Summary		
	1	7	8	9	10	11	14	4	12	2	3	13	4½	Certified	Inspected	Pasteurized Nos. 2, 3, 13
Percentage gas+	40	60	50	30	10	0	100	50	60	10	100	60	10	41.43	55	84
Percentage gas 1–10	40	20	0	30	0	0	20	40	60	30	50	40	0	16.66	50	35
Percentage gas 10+	0	40	50	0	10	0	80	10	0	70	50	20	10	25.82	5	49

The gas formula varied markedly, equal percentages of carbon dioxid and hydrogen occurring when the percentage of gas was high. On storage of the different milks, there was little change in the percentage of samples fermenting lactose with gas formation, except that storage at 37° C. slightly increased the percentage producing gas, and storage at 1° C. decreased it somewhat.

LIQUEFACTION OF GELATIN AND PRODUCTION OF HYDROGEN SULFID AND INDOL.

Breaking up of gelatin is closely allied to protein decomposition and the production of hydrogen sulfid and indol is regarded as positive evidence of putrefaction. Gelatin and sugar-free broth were inoculated with milk when it was received and after storage at different temperatures, so that the presence of protein-digesting organisms might be determined. The results are shown in the following table:

TABLE 8.

SHOWING PERCENTAGE OF MILKS CONTAINING AT THE TIME OF DELIVERY ORGANISMS LIQUEFYING GELATIN AND PRODUCING HYDROGEN SULFID AND INDOL.

	Certified							Inspected		Pasteurized				Summary		
	1	7	8	9	10	11	14	4	12	2	3	13	4½	Certified	Inspected	Pasteurized 2, 3, 13
Gelatin	90	79	84	90	89	58	58	90	88	100	79	79	100	78.0	88	90.0
H_2S	40	50	63	40	60	63	70	50	80	100	50	70	30	54.4	65	73.3
Indol	21	42	42	44	33	0	50	100	88	89	89	100	0	30.3	94	93.0

From this table it will be seen that a large proportion of all milks contained organisms liquefying gelatin and that most samples of inspected and pasteurized milks contained bacteria producing indol, while a small percentage of samples of certified milk contained organisms producing indol. Bacteria producing hydrogen sulfid were found most frequently in pasteurized milks.

On storage at 37° C. the liquefiers of gelatin were crowded out or killed so that the organisms in 1 c.c. of milk no longer produce liquefaction. At room and ice-box temperatures the frequency of these organisms was little affected. When, however, milk was stored for seven to 25 days at 1° C. and then kept in the ice-box for 48 hours, gelatin was liquefied by the bacteria in 1 c.c. of all the supplies.

Organisms producing indol or hydrogen sulfid are little influenced by storage. Generally, however, hydrogen sulfid and indol are more frequently produced by the organisms in 1 c.c. of milk when the milk has been stored at the lower temperatures.

VI. REACTION OF MILK.

1. METHODS AND TECHNIC.

Milk when it is first drawn from the healthy udder of the cow has an acidity which is hardly perceptible; after a very short time the acidity may be readily detected and determined, and usually as milk gets older the acidity increases, gradually causing coagulation.

The acidity of fresh milk as determined with phenolphthalein as an indicator is due principally to the presence of carbonic acid, acid phosphates, ash, and casein. The increase in acidity which appears a very short time after milking is variously ascribed to absorption of carbonic acid, acid phosphates, and calcium compounds. The acidity that develops later is due primarily to the change of lactose into lactic acid, and results from bacterial action, this being dependent on the kinds of bacteria present, their number, and the temperature at which the milk is kept. The change known as coagulation is the result of precipitation of caseinogen by the combined action of acid and calcium salts. The acid is usually lactic and can be titrated, though coagulation also occurs as a result of the formation of amido-acids and possibly also from other causes.[1] Coagulation is regarded as a safeguard because when it occurs it is a visible sign of advancing decomposition of milk.

Acidity determinations of milk are usually made by titrating with a solution of NaOH, phenolphthalein being used as the indicator. These titrations are recorded in different ways: in this country the calculations are made as lactic acid, 1 c.c.of N/1 NaOH neutralizing 0.02 gm. of lactic acid; or also in degrees of acidity, which means number of c.c. of N/10 NaOH per 100 c.c. of milk. In Germany degrees of acidity mean number of c.c. N/4 NaOH per 100 c.c. of milk. Inasmuch as this latter standard was used, all results here indicated will be given in number of c.c. of N/4 NaOH per 100

[1] Babcock and Russell, *Wis. Agric. Exp. Sta.*, 1897, *14th Ann. Rept.*, p. 161, and 1899, *16th Ann. Rept.*, p. 157.

c.c. of milk. For transposition purposes, the following equivalents are given:

1 degree (U.S.) of acidity equals 0.009 per cent lactic acid.

1 degree (German) of acidity equals 0.0225 per cent lactic acid.

1 degree (German) of acidity equals 2.50 per cent (U.S.) acidity.

According to the different observers the acidity of normal fresh milk varies from 4.8 to 8.0 degrees. According to Richmond[1] on boiling milk coagulation occurs if the acidity is above 10.0°; there is a sour taste at 17.6°, and natural curdling occurs at room temperature if the acidity is above 34.0°.

In the work during the past year acidity determinations were made with various objects in view, especially, however, to determine acidity acquired by different grades and classes of milk when kept at different temperatures for varying periods of time, and to learn whether acidity determinations can be used in the establishments of standards for milks.

2. ACIDITY OF DIFFERENT GRADES AND CLASSES OF MILK, WHEN FRESH AND AFTER KEEPING AT DIFFERENT TEMPERATURES FOR VARYING PERIODS OF TIME.

A. FRESH MILK.

Fresh milk is variously reported as having an acidity from 4.8 to 8.0 degrees. Auzinger states that fresh milk from one cow has an acidity of 6.5 to 8.0 degrees, and that the degree of acidity of fresh milk may give valuable information in regard to the cow from which it is obtained:

If the degree of acidity is less than 5.0° suspect mastitis.

If the degree of acidity is between 5.0–6.5° suspect old milkers.

If the degree of acidity is between 8.0–9.0° suspect first or second week after parturition.

If the degree of acidity is above 9.0° suspect colostrum or pathological milk.

Rühm states that milk from a tuberculous udder has an acidity of less than 7°. Hoyberg[2] has devised a method for determining inflammatory processes in the udder of the cow based on the alkaline reaction of serum exuded in inflammatory processes, using rosolic acid in alcoholic solution as an indicator. He found that the decreased acid reaction is not proportionate to number of bacteria

[1] *Analyst*, 1900, 25, p. 116.

[2] *Ztschr. f. Fl. u. Milch-Hyg.*, 1911, 21, p. 133.

or leukocytes present in freshly drawn milk, but is proportionate to the exudation of serum.

a) *Reaction of freshly drawn milk.*—A number of determinations of acidity of milk within 15 minutes after drawing were made. Samples were taken from one quarter of the udder at the beginning, middle, and end of milking. The average results obtained were 7.0° of acidity at the beginning, 5.8° in the middle, and 6.6° at the end.

Likewise experiments were tried to test out the method of Hoyberg. His reagent is made as follows: 1 per cent alcohol solution of rosolic acid in 96 per cent alcohol in proportions of 0.45 c.c. to 5 c.c. Use 5 c.c. of milk and 5.5 c.c. of the reagent.

	Healthy Cows	Just Calved		Mastitis Streptococcus	Old Caked Udder
		1 Qt. Caked	Clear		
Average reaction	6.1°	7.75°	7.0°	2.62°	5.45°
Rosolic acid solution	Yellow	Yellow	Yellow	Deep red	Light red
Average Tromsdorff reading	5/16	0	1/4	Very much	1 1/2

Because of the gradation of colors from yellow to red it is hard to detect slight changes in reaction by Hoyberg's method. Moreover, the test must be applied soon after milking, because otherwise acidity due to bacterial action overcomes decreased acidity due to inflammation.

b) *Reaction of different grades and classes of milk as received in regular delivery.*—Milk as it is delivered to consumers in our larger cities is from six to 60 hours old. Refrigeration and pasteurization will retard the development of acid-producing organisms.

Based on a large number of observations during the year the average, maximum, and minimum degrees of acidity as milk is received are shown in the following table:

TABLE 9.

SHOWING ACIDITY OF MILK AS IT IS RECEIVED.

	Certified							Inspected		Pasteurized			
	1	7	8	9	10	11	14	4	12	2	3	13	4 1/2
Average acidity	6.006	5.98	5.95	6.26	6.38	6.37	6.6	6.23	6.41	5.69	5.97	6.36	6.08
Maximum	7.0	7.0	7.0	7.4	7.6	8.0	7.2	8.0	6.8	8.0	7.6	8.2	6.2
Minimum	4.8	5.2	4.4	5.6	5.2	5.2	6.0	5.2	6.0	4.0	5.2	5.2	6.0
Average	Certified 6.18							Inspected 6.306		Pasteurized 5.96			

From Table 9 it will be seen that the lowest degree of acidity was observed in pasteurized milk, and that the same maximum degree observed was found in all three grades. While the variations are marked in all grades and in nearly all supplies, still the entire average for pasteurized market milks was lowest, and for inspected highest, while certified milk stood midway between. This in itself is rather good evidence that the four pasteurized milks are not spoiled milks pasteurized for the purposes of redemption.

B. ACIDITY OF MILK WHEN KEPT FOR VARYING PERIODS OF TIME AT CERTAIN TEMPERATURES.

The changes in acidity which occur in milk when it is kept for a period of time are of more importance than is generally recognized.

TABLE 10.

SHOWING GREATEST VARIATIONS IN ACIDITY BEFORE COAGULATION WHEN MILK IS STORED AT DIFFERENT TEMPERATURES.

CLASS	WHEN RECEIVED	37° C.	ROOM TEMP.		ICE-BOX TEMP.		1° C.		
		12 Hrs.	24 Hrs.	48 Hrs.	24 Hrs.	72 Hrs.	7 Days	14 Days	25 Days
Certified	4.4 to 8.0	6.4 to 19.6	5.4 to 7.3	5.4 to 14.6	6.0 to 7.4	6.4 to 9.9	5.6 to 7.9	0.8 to 7.6	8.4 to 13.8
Inspected	5.2 to 8.0	16.2 to 27.2	5.9 to 7.0	...	7.2 to 7.5	7.2 to 8.9	5.9 to 7.4	7.0 to 13.4	12.1 to 23.4
2, 3, 13 pasteurized	4.0 to 8.2	20.0 to 24.2	6.8 to 10.4	26.0	5.8 to 13.6	. .	6.0 to 8.0	7.0 to 9.8	13.5 to 24.2
4½ pasteurized	6.2 to 6.2	12.2	6.0	....	6.0	6.8	6.2	6.8 to 7.2	7.6 to 11.8

TABLE 11.

SHOWING AVERAGE ACIDITY BEFORE COAGULATION WHEN MILK IS STORED AT DIFFERENT TEMPERATURES.

CLASS	WHEN RECEIVED	37° C.	ROOM TEMP.		ICE-BOX TEMP.		1° C.		
		12 Hrs.	24 Hrs.	48 Hrs.	24 Hrs.	72 Hrs.	7 Days	14 Days	25 Days
Certified	6.18	11.3	6.4	8.5	6.7	7.7	7.6	6.6	10.0
Inspected	6.306	22.2	6.4	.	7.4	8.1	6.75	8.1	16.3
2, 3, 13 pasteurized	5.96	24.7	9.0	26.0	8.5	.	7.1	8.3	18.3

These changes indicate activity and presence of types of micro-organisms and ferments as well as give to the consumer a warning of the unfitness of the milk for household use. One of the objections

to pasteurized milk is that it does not sour or coagulate because the acid-producing organisms have been destroyed by heat, thus removing one of nature's danger signals.

a) *Reaction after varying periods of storage at different temperatures before coagulation occurs.*—The acidity in milk generally increases on storage, ultimately causing coagulation. To determine the rate of acidification when it is stored at different temperatures, bottles of milk were stored at 37° C., at room temperature, at ice-box temperature, and at a point just above freezing. Examinations were made at regular intervals before and at the time when coagulation occurred. The results of these investigations are shown in Tables 10 and 11.

From these tables it is evident that the acidity of pasteurized milks increases more rapidly and is more marked than that of raw milk. At the higher temperatures the acidity is not only more quickly but more markedly increased than at the lower temperatures.

b) *Time required for clotting.*—At different times milks were stored at definite temperatures and observations made on the length of time required for coagulation and for changes in reaction.

TABLE 12.

SHOWING WHEN CLOTTING APPEARED ON STORAGE AT DIFFERENT TEMPERATURES.

Temp.	Certified							Inspected		Pasteurized			
	1	7	8	9	10	11	14	4	12	2	3	13	4½
37° C.	36–72 hrs.	24–48 hrs.	12–72 hrs.	24–48 hrs.	24–48 hrs.	48–120 hrs.	12–72 hrs.	12–48 hrs.	24–120 hrs.	12–48 hrs.	12–48 hrs.	12–96 hrs.	12–48 hrs.
Room	3–11 days	3–6 days	2–6 days	2–4 days	3–4 days	3–6 days	3–7 days	2–4 days	2–4 days	3–4 days	2–4 days	2–5 days	1–3 days
Ice-box	5–22 days	6–21 days	6–22 days	6–9 days	6–19 days	7–14+ days	22+–50 days	7–9 days	6–9 days	5–9 days	4–9 days	6–8 days	6–8 days
1° C.	20–37 days	31–46 days	31–39 days	21–34 days	16–66 days	25–53 days	33+–60 days	21–49 days	21–39 days	20–40 days	14–37 days	21–53 days	60+ days

TABLE 13.

SHOWING THE GREATEST DIFFERENCES IN TIME AFTER DELIVERY AT WHICH COAGULATION OCCURRED IN THE DIFFERENT CLASSES OF MILK WHEN KEPT AT DEFINITE TEMPERATURES.

Temp.	Certified	Inspected	Pasteurized
37° C.	12–120 hrs.	12–120 hrs.	12–96 hrs.
Room	2–11 days	2–4 days	1–5 days
Ice-box	5–50 days	6–9 days	4–9 days
1° C.	16–60 days	21–49 days	14–60 days

TABLE 14.

SHOWING THE AVERAGE TIME AT WHICH COAGULATION OCCURS.

Temp.	Certified							Inspected		Pasteurized			
	1	7	8	9	10	11	14	4	12	2	3	13	4½
37° C.	49.3 hrs.	41.14 hrs.	48.8 hrs.	30.8 hrs.	34.3 hrs.	61.7 hrs.	40.8 hrs.	24 hrs.	51.4 hrs.	24 hrs.	23.45 hrs.	42.8 hrs.	24 hrs.
Room	5.0 days	4.16 days	4.85 days	3.14 days	3.25 days	4.57 days	5.75 days	3.125 days	3.0 days	3.125 days	3.0 days	3.5 days	2.75 days
Ice-box	14.6 days	10.7 days	11.7 days	7.37 days	9.1 days	12.4 days	37.0 days	8.125 days	7.57 days	7.25 days	7.125 days	6.87 days	6.75 days
1° C.	32.2 days	36 days	33.75 days	31.3 days	36.4 days	36.5 days	50.0 days	33.8 days	29.5 days	32.14 days	30.4 days	35.0 days	60+ days

TABLE 15.

SHOWING THE AVERAGE TIME REQUIRED AFTER DELIVERY FOR COAGULATION OF THE DIFFERENT GRADES OF MILK AT DIFFERENT TEMPERATURES.

Temp.	Certified	Inspected	Pasteurized
37° C.	44.2 hrs.	36.8 hrs.	28.7 hrs.
Room	4.28 days	3.07 days	3.14 days
Ice-box	11.82 days	7.86 days	6.93 days
1° C.	34.07 days	32.1 days	28.55 days

From Tables 12–15 it is evident that at the temperatures indicated, 37° C., room, ice-box, and just above the freezing point, coagulation occurs in pasteurized milk as soon after delivery as in good raw milks. Moreover, certified milks do not coagulate nearly as soon as inspected and pasteurized milks. At low temperatures coagulation of certified, inspected, and pasteurized milks occurs late, and, as will be shown subsequently, these milks may have a high bacterial content long before they coagulate. Since coagulation is looked upon as the visible means of determining when milk is no longer fresh, these facts are important.

Realizing that if milk is to be kept by the dealer for any length of time before selling the storage temperature must be low, and the

TABLE 16.

SHOWING NUMBER OF DAYS REQUIRED AT 50–60° F. FOR COAGULATION AFTER STORAGE NEAR THE FREEZING POINT.

Days at 1° C.	Certified							Inspected		Pasteurized			
	1	7	8	9	10	11	14	4	12	2	3	13	4½
7 days	6	3	3	4	5	10	10	..	6	6	6	3	4
15 days	11	3	2	4	3	6	15	3	4	3	5	4	4
25 days	1	1	..	1	1	3	3	1	1	1	1	3	9

TABLE 17.

SHOWING MAXIMUM AND MINIMUM TIMES AT 50–60° F. REQUIRED FOR COAGULATION OF MILK PREVIOUSLY STORED FOR VARYING PERIODS OF TIME AT LOW TEMPERATURE.

Days at 1° C.	Certified	Inspected	Pasteurized	Ocean Shipments
7	3–10 days	6 days	3–6 days	5–15 days
15	2–15 days	3–4 days	3–5 days	4–15 days
25	1–3 days	1 day	1–9 days	8–20 days

temperature in the household will at best be that of the ice-box (50–60° F.), an attempt was made to simulate these conditions; the various milks were stored at 1° C. for varying periods of time and then kept at ice-box temperature to determine when clotting occurs.

From Tables 16 and 17 it is evident that milks stored at low temperatures for varying periods of time coagulate more rapidly at the ordinary ice-box temperature than do fresh milks.

c) *Reaction when clotting occurs at different temperatures of storage.*—On aging, milk no longer consists of cream and skim milk only, but undergoes further separation. Coagulation being due to increased acidity, the reaction of the whole mixture was determined at the time of coagulation (Tables 18–21).

TABLE 18.

SHOWING IN DEGREES REACTION OF WHOLE MIXTURE WHEN COAGULATION OCCURS.

Temp.	Certified							Inspected		Pasteurized			
	1	7	8	9	10	11	14	4	12	2	3	13	4½
37° C.	23.3 to 36.8	22.0 to 48.0	30.0 to 40.0	19.6 to 52.8	24.0 to 46.0	28.0 to 36.0	18.0 to 64.0	27.2 to 89.6	26.0 to 31.8	20.0 to 84.0	24.2 to 76.0	22.4 to 86.0	12.2
Room	28.0 to 42.0	11.0 to 56 0	22.0 to 38.0	24.0 to 38.0	24.0 to 44.0	24.0 to 38.0	10.6 to 28.0	23.8 to 44.0	28.4 to 44.0	26.0 to 60.0	25.6 to 78.0	24.0 to 80.0	6.0
Ice-box	8.0 to 30.0	10.0 to 36.0	24.0 to 28.0	26.0 to 38.0	10.0 to 42.0	18.0 to 33.0	16.0 to 34.6	16.0 to 36.0	24.0 to 38.0	24.0 to 31.7+	24.0 to 38.0	26.0 to 38.0	13.4
1° C.	7.2 to 17.6	25.2 to 34.0	8.0 to 30.0	24.4 to 42.0	20.0 to 40.0	12.0 to 16.6	11.8 to 13.6+	24.0 to 40.0	20.0 to 34.0	26.0 to 40.0	12.0 to 40.0	24.0 to 36.4	...

It is evident from the varying degrees of acidity at which coagulation occurs that acid, at least as determined by titration with NaOH and phenolphthalein, is not the only factor in coagulation. Apparently ferments, peptonization, etc., play some part also.

TABLE 19.

SHOWING GREATEST VARIATIONS IN ACIDITY AT WHICH COAGULATION OCCURRED AT DIFFERENT TEMPERATURES.

Temp.	Certified	Inspected	Pasteurized
37° C.	18.0–52.8	26.0–89.6	20.0–86.0
Room	10.6–56.0	23.8–44.0	6.0–80.0
Ice-box	8.0–42.0	10.0–38.0	13.4–38.0
1° C.	7.2–42.0	20.0–40.0	12.0–40.0

TABLE 20.

SHOWING AVERAGE ACIDITY WHEN COAGULATION OCCURRED AT DIFFERENT TEMPERATURES.

Temp.	Certified							Inspected		Pasteurized			
	1	7	8	9	10	11	14	4	12	2	3	13	4½
37° C.	30.6	37.84	32 5	37.4	35 95	42.85	33 05	50.82	30.95	54 62	45.4	51.28	12.2
Room	34.6	43 75	28.6	31 3	33.95	29.65	20.15	32.45	34.13	39.48	43.9	45.12	6.0
Ice-box	17.1	20 12	25.88	34.72	29.77	25 25	25.3	28.84	28.91	29.0	29.34	30.6	13.4
1° C.	11.2	29.06	19.35	30.06	29 5	14 15	11.8	35.70	26.26	34.76	29.44	30.13	....

TABLE 21.

SHOWING AVERAGE ACIDITY OF DIFFERENT GRADES OF MILK WHEN COAGULATION OCCURS.

Temp.	Certified	Inspected	Pasteurized
37° C.	34.19	44.51	48.77
Room	30.08	33.17	40.31
Ice-box	26.00	28.88	28.78
1° C.	22.85	31.11	31.87

Particular samples of each milk show that the reaction when coagulation occurs varies greatly. The average reaction for all milks at the time of coagulation is highest when kept at 37° C., next highest when kept at room temperature, and lowest at the ice-box temperature except for certified milks. Certified milk coagulates with less acidity than does any other milk, but more time is required, as shown in Table 15.

d) Reaction of different parts of milk physically changed.—Milk, when kept, usually coagulates or clots, and whey becomes separated. These changes are generally due to the production of lactic acid from lactose. Coagulation, however, is not the only physical change observed, for in almost all milks there are also nitrogenous changes which are generally referred to as peptonization and putrefaction. These are by some supposed to make milk alkaline. That this seldom happens will be shown later. What does happen, however, is that milk in which there has been much protein change

is less markedly acid than is other milk. When a yellow fluid forms under the cream it is generally regarded as an evidence of peptonization.

Determinations were made of the reaction of different parts of milk which had undergone coagulation or peptonization.

TABLE 22.

SHOWING AVERAGE REACTION OF DIFFERENT PARTS OF MILK PHYSICALLY CHANGED AS A RESULT OF STORAGE AT DIFFERENT TEMPERATURES.

Storage Temp.	Average Days Kept	Certified			Inspected			Pasteurized		
		Whey	Pep. Fl.*	Mixed	Whey	Pep. Fl.	Mixed	Whey	Pep. Fl.	Mixed
37° C.	7	20.0	29 05	37.4	37.0	33.0	39.3	54.4	51.3	57.3
Room	20	48.0	32.1	42.49	....	32.7	42.7	45.0	46.6	51.46
Ice-box	19	....	22.77	28.6	....	27.3	44.0	....	24.85	36.0
1° C.	34	29.75	13.3	28.19	38.0	30.0	38.0	27.2	28.0	34.6

* Peptonized fluid.

TABLE 23.

SHOWING AVERAGE REACTION OF DIFFERENT PARTS OF CERTIFIED, INSPECTED, AND PASTEURIZED MILKS PHYSICALLY CHANGED AS A RESULT OF STORAGE.

	Whey	Peptonized Fluid	Mixed
Certified	30.54	24.74	33.7
Inspected	37.50	31.33	40.8
Pasteurized	38.62	33.13	40.95
Total average	37.23	28.34	36.44

TABLE 24.

SHOWING AVERAGE REACTION OF DIFFERENT PARTS OF MILKS PHYSICALLY CHANGED AT DEFINITE TEMPERATURES.

Temp.	Whey	Peptonized Fluid	Mixed
37° C.	40.56	33.64	41.05
Room	37.20	30.11	39.98
Ice-box	.....	23.20	31.68
1° C.	30.0	19.6	31.68

From these tables it is evident that the straw-colored (peptonized) fluid which forms under the cream after milk has been stored for some time is less acid than the whey or whole mixture. That the curd contains much acid is shown by the fact that a mixture of the entire quantity is more acid than either the whey or peptonized fluid. This latter conclusion agrees with that of Farrington.[1]

[1] *Wis. Agric. Exp. Sta.*, 1903, *20th Ann. Rept.*, p. 134.

Reaction of cream: Top milk (gravity cream) freshly obtained, or after being stored for several days at a low temperature, is of the same reaction as the skimmed milk below. After storage at ice-box, room, or incubator temperatures for a relatively short period of time, a difference in the acidity of gravity cream and skimmed milk is observed. This is to be expected, for acidity develops only from the serum containing lactose. Farrington emphasizes the fact that the amount of serum present in cream must be taken into consideration when acidity of different samples of cream are compared. To do this he advocates the adoption of the acidity of cream of given richness as a standard, suggesting 0.6 per cent lactic acid in cream containing 25 per cent of fat.

Numerous tests of acidity of gravity cream and skimmed milk were made on delivery of the various milks, and it was found that quite constantly only about five-sevenths as much sodium hydroxid was necessary to neutralize the same amount of cream as of skimmed milk. Acidity determinations of the cream, skimmed and well mixed milks were made after 14 and 25 days of storage at 1° C., and after 14 and 25 days at 1° C. followed by 48 hours of storage at the ice-box temperature. The results are shown in the following table:

TABLE 25.

SHOWING AVERAGE NUMBER OF CUBIC CENTIMETERS OF N/4 NaOH NECESSARY TO NEUTRALIZE 100 C.C. OF CREAM, SKIMMED AND MIXED MILK AFTER STORAGE.

Storage	Certified			Inspected			Pasteurized (2, 3, 13)		
	Cream	Skimmed	Mixed	Cream	Skimmed	Mixed	Cream	Skimmed	Mixed
14 days at 1° C.	10.3	8.0	8.7	16.8	27.8	25.6	17.1	13.3	14.6
25 days at 1° C.	12.9	9.3	9.3	15.0	18.6	18.8	26.1	38.0	37.0
48 hrs. at 15° C. after 14 days at 1° C.	12.7	10.9	11.5	21.5	77.0	23.6	35.7	36.3	36.8
48 hrs. at 15° C. after 25 days at 1° C.	17.9	25.0	24.6	18.6	28.8	29.8	28.6	46.7	43.4

From this table it will be seen that until there is considerable acidity in the mixed milk the gravity cream is as acid or more acid than the skimmed milk. This holds true of the three classes of milk studied, until the acidity reaches 18°. Whether good milk stored at higher temperatures gives the same reactions I have not determined, nor did I determine whether the results will be the same in separator cream and skim milk stored separately. Aging

of separator cream is resorted to commercially to make cream appear rich.

e) *Ultimate changes.*—Most milks coagulate naturally with an increase of acidity on storage. Of all the many samples of milks examined only 19 became less acid, and only one became actually alkaline in reaction. Coagulation is, however, not universal, for 42 samples of milk kept at 1° C. did not coagulate after periods of time from one to 10 months. This occurred most frequently in certified and the higher grades of pasteurized milks. In these, changes were evidenced by effervescence when the bottle was opened or by odors indicating protein decomposition. Decomposition without coagulation is of great importance, for by many people milk is considered suitable for human consumption until curdling occurs. While in most cases curdling precedes marked protein changes, still putrefaction may take place and curdling never appear.

Physical changes in milk stored at a definite temperature vary to some extent for different milks, for the same milks, and for different bottles of one supply taken on the same day. In spite of these variations certain changes in physical appearance and reaction may usually be depended on for each grade of milk.

Physical changes: From these changes some of the chemical changes that have occurred may be recognized. The ultimate changes shown in the following table are based on observations made on 380 bottles of milk, of which 64 were stored 220 days at 37° C., 106 for 270 days at room temperature, 86 for 250 days at ice-box temperature, and 124 for 250 days at 1° C.

TABLE 26.

SHOWING PHYSICAL CHANGES OCCURRING IN MILK STORED 220 TO 270 DAYS.

	37° C.			ROOM			ICE-BOX			1° C.		
	Certified	Inspected	Pasteurized	Certified	Inspected	Pasteurized	Certified	Inspected	Pasteurized	Certified	Inspected	Pasteurized
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
No coagulation	0	0	0	0	0	0	6	0	0.0	20	20	7
Solid clot	0	0	0	0	0		0	0	6.5	9	20	23
Clot and clear whey	5	50	0	61	71	22	23	60	32.0	41	40	35
Eroding clot	85	50	100	21	29	61	48	40	65.0	18	20	35
Clot disappearing	10	0	0	18	0	17	23	0	0.0	12	0	0
Putrid without clot	0	0	0	0	0	0	6	0	0.0	20	20	7

From Table 26 it is seen that pasteurized milk is likely to undergo solution of the clot, and that in certified milk complete solution is frequently seen. Coagulation without separation of whey occurs only when milk is refrigerated. Putrefaction without coagulation occurs principally when milks are stored at low temperatures.

When milk is frozen, there is separation into two zones under the cream. Of these, the upper zone is usually translucent, grayish white, while the lower zone is flocculent. A number of bottles of the different supplies received were kept below the freezing point for almost nine months. When these were thawed out the normal appearance was soon re-established, but examination showed that the milks had deteriorated.

Reaction: Inasmuch as the change in reaction of milk is due largely to the action of bacteria, we expect changes of reaction until all food, the metabolism of which influences reaction, is consumed, and until the acid-producing bacteria are destroyed or inhibited in action due to their own products, and the counter-influences on reaction have become stable.

Generally marked acidification is produced if milk is stored a long time, although actual alkalinity or decrease in acidity sometimes is observed. The following table shows the average, maximum, and minimum acidity in degrees for the same 380 bottles stored for periods from 220 to 270 days:

TABLE 27.

SHOWING AVERAGE MAXIMUM AND MINIMUM ACIDITY RESULTING AFTER LONG STORAGE.

	37° C.			Room			Ice-Box			1° C.		
	Certified	Inspected	Pasteurized	Certified	Inspected	Pasteurized	Certified	Inspected	Pasteurized	Certified	Inspected	Pasteurized
Average	93.5	102.3	63.8	78.8	155.7	135.98	60.6	90.5	92.2	33.1	35.8	40.6
Highest	124.8	120.0	109.2	171.2	192.8	203.2	118.4	136.4	159.6	48.0	40.4	54.0
Lowest	Alk.	72.4	3.6	8.0	13.6	56.4	20.0	60.0	55.2	15.0	31.2	29.6

It will be seen from this table that at room temperature the highest acidity is developed, that at 37° C. no alkaline reaction is produced, and that at the lower temperatures acidity is always increased when milk is stored for a long period of time.

Some milks stored at 1° C. for periods varying from six to 10 weeks remained unchanged in appearance. In these the following reactions were observed:

TABLE 28.

Showing Average Maximum and Minimum Acidity after Six to Ten Weeks' Storage at 1° C.

	Certified	Inspected	Pasteurized
Average	29.6	33.5	19.9
Highest	51.8	49.8	43.8
Lowest	9.6	24.0	0.8

From this it is seen that pasteurized milk not coagulating on storage at very low temperature acidifies less than raw milk; it also decreases in acidity more frequently during the first months of storage but later becomes as acid as raw milk. When pasteurized milks are kept at room temperature for long periods of time, as is seen from Table 27, they at times become very acid, exceeding the maximum of 0.8 per cent lactic acid, or 35.5°, beyond which Farrington states the acidity will not go.

f) Alkaline milk.—Alkaline milk is frequently referred to, and its occurrence might seem not unusual. Observations of a large number of samples of milk stored for varying periods of time at various temperatures lead me to believe that partial neutralization of the original acidity in fresh milk is infrequent and actually alkaline milk is very rare. While many milks contain organisms which, if acting alone, will produce alkalies, still these milks also contain organisms breaking up the lactose in milk and so there is an increased acidity. Furthermore it has been known for a long time, and has been emphasized more recently by Kendall, that organisms break up the carbohydrates before they act on the proteins, and organisms growing together do not always produce the same end results they produce when grown alone. These mixtures of bacteria in a medium containing the various food materials do not always produce end results representing the sum of their products but rather the products of symbiotic action. Investigations of alkaline milk and of reduction of acidity are shown in Table 29.

This table shows plainly how seldom the acidity of milk is actually decreased on aging and further shows how very rare actual

alkalinity is. It was, however, noticed that if acidity determinations of stored milks are made at frequent intervals, the acidity of some samples at first increases and then gradually decreases. It would seem fair to conclude that in such milks there is alkaline production even though the reaction is still acid.

TABLE 29.

SHOWING OCCURRENCE OF ALKALINE MILK AND REDUCTION OF ACIDITY WHEN MILK IS STORED AT DIFFERENT TEMPERATURES FOR PERIODS VARYING FROM 12 HOURS TO 270 DAYS.

	ALKALINE MILK					MILK WITH DECREASED ACIDITY				
	37° C.	Room	Ice-Box	1° C.	Frozen	37° C.	Room	Ice-Box	1° C.	Frozen
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
Certified	3.4	0	0	0	0	0.0	5.5	8.5	7.1	28.5
Inspected	0.0	0	0	0	0	0.0	10.0	0.0	0.0	0.0
Pasteurized, 2, 3, 13	0.0	0	0	0	0	8.5	0.0	0.0	14.7	0.0

g) *Odor of milk on aging.*—Next to coagulation the odor of milk is the most accessible test of its fitness for use. Fresh clean milk has very little odor, but as changes occur odors and flavors of different kinds result. These odors are of considerable importance in showing the character of changes, and so were noted carefully in a large number of cases. The odors observed when milks coagulate and after long storage are given in Tables 30 and 31.

These tables show that when milks coagulate at 37° C. most of them have a lactic acid odor, and that there is little putrefaction, but after long storage the strong odors of butyric acid and putrefaction become more prominent. At lower temperatures of storage putrefactive odors are not preceded by the usual odor of sour milk

TABLE 30.

SHOWING PERCENTAGE OF OCCURRENCE OF DIFFERENT ODORS AT THE TIME OF COAGULATION.

TEMP. OF STORAGE	CERTIFIED						INSPECTED						PASTEURIZED, 2, 3, 13					
	Acid						Acid						Acid					
	Lactic	Acetic	Butyric	Rancid	Putrid	Fermented	Lactic	Acetic	Butyric	Rancid	Putrid	Fermented	Lactic	Acetic	Butyric	Rancid	Putrid	Fermented
37° C.	68.8	0	7.0	4.9	7.2	11.9	86.6	0	13.4	0	0	0.0	81.6	0	13.6	0.0	4.8	..
Room	43.8	0	31.5	4.9	7.2	12.6	27.3	0	54.6	0	0	18.1	56.4	0	34.7	0.0	4.4	..
Ice-box	4.9	0	26.4	11.9	39.1	17.7	45.4	0	45.4	0	0	9.2	56.0	0	33.3	16.6	16.6	..
1° C.	4.4	0	13.1	17.3	65.2	0.0	0.0	0	44.4	0	0	0.0	7.1	0	14.2	64.5	0.0	..

TABLE 31.

SHOWING PERCENTAGE OF OCCURRENCE OF DIFFERENT ODORS WHEN MILKS ARE STORED FOR PERIODS VARYING FROM 220 TO 270 DAYS.

Temp. of Storage	Certified						Inspected						Pasteurized, 2, 3, 13					
	Acid						Acid						Acid					
	Lactic	Acetic	Butyric	Rancid	Putrid	Fermented	Lactic	Acetic	Butyric	Rancid	Putrid	Fermented	Lactic	Acetic	Butyric	Rancid	Putrid	Fermented
37° C...	0.0	0.0	26.3	26.3	31.6	15.8	20.0	0.0	0.0	40.0	40.0	0.0	0.0	10.0	0.0	10.0	60.0	21 0
Room..	0.0*	16.8	8.4	12.5	41.6	16.8	0.0	37.5	25.0	0.0	12 5	25.0	0.0‡	30.9	15 4	23 1	23.1	0.0
Ice-box	5.2	0.0	15.6	15.6	46.1	17.5	7.2	0.0	14 3	14.3	35.7	28.5	0.0	12.5	0.0	31 2	31 2	25 1
1° C....	10.0	5.0	7.5	7.5	32.5	37.5	†	....	....	....	....	....	14.2	0.0	14.2	42.4	14.6	14.6

* Fatty acid in 3.9 per cent of samples.

† Too few observations to warrant percentage estimates.

‡ Fatty acid odor in 6.6 per cent of samples.

and inasmuch as the detection of these odors when not marked is made only by those properly trained, milks that putrefy without souring are likely to be used when unfit. Putrefaction without souring occurs more frequently in certified than in inspected and pasteurized milks.

3. METHODS FOR READILY DETECTING INCREASED ACIDITY.

Reaction, physical appearance, and odor are of considerable importance as indicators of the fitness of milk as a food. In the home and dairy, physical appearance, clotting on boiling, and odor are relied on; while in the laboratory, determinations of the reaction are added to these indicators. Milk as it is offered for sale in our larger cities is seldom near the souring point as determined by taste and smell, nor have putrefactive changes gone on to the extent of being perceptible by these senses. No milks of which daily deliveries were received at the laboratory had deteriorated sufficiently for one to detect decomposition by taste or smell. Before coagulation occurs increased acidity can be determined by titration in the laboratory. This requires time and for this reason titration cannot be used to prevent the sale of soured milk.

Rapid and convenient methods of detecting increased acidity have been recommended. The use of Farrington's alkaline tablet is one of these, and gives the reaction with phenolphthalein as the indicator. At dairies where milk is received for cheese and butter

making the alizarin test of Engling is used, but it is of value only in the detection of advanced acidity. Rühm has recommended a test for detection of beginning acidification in mixed milks of two or more cows. According to this method 10 c.c. of 68 per cent alcohol are added to 10 c.c. of the milk to be tested. If there is immediate coagulation the acidity is above 8.0°. This test I have tried on a large number of samples and found that the 68 per cent alcohol must be made from absolute alcohol. More advanced acidity is detected by boiling a small amount of milk for a few moments in a test tube. According to Rühm, coagulation on boiling appears if the acidity is above 10.0°.

No milk received at the laboratory coagulated when an equal amount of 68 per cent alcohol was added. The acidity of the various milks as received was from 4.8° to 8.2°. The alcohol and heating methods were also tried on milks stored at different temperatures, the results being shown in Tables 32 and 33.

TABLE 32.

SHOWING VALUE OF 68 PER CENT ALCOHOL AS A MEANS OF DETERMINING BEGINNING ACIDIFICATION, THERE BEING NO COAGULATION ON BOILING.

TEMP.	CERTIFIED				INSPECTED				PASTEURIZED, 2, 3, 13			
	Average Coagulating	Highest Coagulating	Lowest Coagulating	Highest Not Coagulating	Average Coagulating	Highest Coagulating	Lowest Coagulating	Highest Not Coagulating	Average Coagulating	Highest Coagulating	Lowest Coagulating	Highest Not Coagulating
37° C.	9.8	11.1	9.0	...	...	...	...	...	...	...	...	...
Room	8.2	10.6	7.3	7.0	...	...	...	7.0	...	...	8.2	6.0
Ice-box	7.8	9.9	6.8	7.4	8.9	9.8	8.1	7.7	9.0	7.7	8.0	7.2
1° C	9.5	13.4	7.2	7.6	8.3	8.5	7.9	7.5	8.4	9.8	7.3	7.6

Total average acidity at which coagulation with 68 per cent alcohol occurs equals 8.54°. Highest acidity observed which did not cause coagulation with 68 per cent alcohol equals 7.7°. Lowest acidity observed which caused coagulation with 68 per cent alcohol equals 6.8°.

It will be noticed that by this test lower acidification is detected than by the senses of smell and taste, or by boiling. All samples of milk coagulating on boiling coagulated also on the addition of an equal part of 68 per cent alcohol.

The method most certainly is of value in detecting increase in acidity. Higher percentages of alcohol were tried but were not as satisfactory as the 68 per cent solution.

TABLE 33.

SHOWING VALUE OF BOILING MILK AS A MEANS OF DETERMINING ADVANCED ACIDITY, NORMAL CLOTTING NOT HAVING OCCURRED.

Storage Temp.	Certified				Inspected				Pasteurized			
	Average Coagulating	Highest Coagulating	Lowest Coagulating	Highest Not Coagulating	Average Coagulating	Highest Coagulating	Lowest Coagulating	Highest Not Coagulating	Average Coagulating	Highest Coagulating	Lowest Coagulating	Highest Not Coagulating
37° C.	23.7	37.8	13.8	11.1	21.6	29.2	16.2	...	21.3	28.8	12.2	...
Room	18.1	33.8	11.0	10.6	26.6	28.4	23.8	7.0	15.2	25 6	9.8	8.2
Ice-box	9.2	10.6	8.4	9.9	....	....	....	...	13.5	13.6	13.2	9.7
1° C.	10.9	13.8	9.0	13.4	13.4	13.4	11.5	8.5	18.8	11.8	17.2	9.8

Total average acidity at which coagulation on boiling occurs equals 16.2°; highest acidity observed which did not cause coagulation on boiling equals 13.4°; lowest acidity observed which caused coagulation on boiling equals 8.4°.

Acidity and heat are apparently not the only factors in coagulation, as is evident from the variation in acidity when coagulation occurs as a result of boiling. Similar results have been obtained by Stokes,[1] Richmond, and others. Boiling is essentially a home test and by it suspected acidification can be confirmed.

VII. FERMENTS IN MILK.

Ferments or enzymes are usually present in milk. Their importance has been emphasized especially since the introduction of pasteurization. Preservation of enzymes has probably been the most potent factor in reducing the temperature for pasteurization. Many different kinds of enzymes in milk have been described, as galactase, amylase, lipase, peroxidase, catalase, salol-splitting ferments, etc. The original galactase of Babcock and Russell (*loc. cit.*) has been found by Wender[2] to consist of galactase proper, catalase, and peroxidase. Various tests have been devised for detecting the presence of these enzymes. While it is impossible to go into detail in regard to the methods and value of all these tests, a few

[1] *Analyst*, 1900, 16, p. 122.   [2] *Oesterr. Chem. Ztschr.*, 1903, 7, p. 1.

are readily made and give much information. Milk ferments are regarded of importance especially in the feeding of infants.[1]

Inasmuch as ferments are produced by bacteria, some discussion has arisen in regard to the origin of the ferments in milk. There now can be no doubt that some ferments may be naturally present in milk, these coming from the cow, while others are produced by the bacteria. To distinguish between and determine the amounts of these ferments tests have been devised, and efforts have been made to ascertain the bacterial content of milk from the amount and activity of its ferments.

The tests used most frequently and successfully were those for catalases and reductases. The following methods were used:

TESTS USED.

CATALASE TEST.

In Lobeck's tube 15 c.c. of the milk to be tested and 5 c.c. of 3 volume per cent hydrogen peroxid were mixed and the stopper tightly inserted. The tube for measuring the liberated oxygen was then filled with water and inserted in the hole in the stopper, pushing out the small hard rubber button. The mixture of milk and hydrogen peroxid was immersed up to the stopper in a water bath at 37° C. and left there for two hours. The oxygen liberated replaced the water in the graduated tube after which the readings were made. The method is similar to that devised by Rühm and Auzinger, except that they used 10 c.c. each of milk and hydrogen peroxid. I used larger quantities of milk and less hydrogen peroxid so as to get satisfactory readings for pasteurized milk. According to Auzinger much gas occurs (1) with physiologically changed milk as is the case with colostrum and with milk from old milkers, (2) with pathologically changed milk as in mastitis and other febrile diseases, or (3) with bacteria rich milk.

REDUCTASE TESTS.

a) *Schmidt-Müller or slow reductase test.*—The reagent is made by adding 195 c.c. of distilled water to 5 c.c. of saturated alcoholic solution of methylene blue (zinc chlorid double salt). This reagent should be boiled every day before using. The test is made by adding to 20 c.c. of milk in a test tube 1 c.c. of the reagent,

[1] Freeman, *Jour. Am. Med. Assoc.*, 1907, 49, p. 1740.

mixing, sealing with melted paraffin, and then incubating at 45° C. in a water bath. According to Rühm fresh milk remains blue for 12 hours or more, and infected milk decolorizes in less than one hour. Reductases, according to Rühm, are increased by acid formers but not by alkaline producers. Auzinger, quoting from Jensen, who uses 0.5 c.c. of the reagent in 20 c.c. of milk, states that on holding the mixture at 38° to 40° C. milk not decolorizing in seven hours contains less than 100,000 bacteria per cubic centimeter, that which decolorizes in two to seven hours contains 100,000 to 300,000, and that which decolorizes in one-fourth to two hours contains 300,000 to 20,000,000 bacteria per cubic centimeter.

b) Schardinger or hastened reductase test.—The reagent is made by adding 190 c.c. of distilled water and 5 c.c. of formaldehyde solution to 5 c.c. of saturated alcoholic solution of methylene blue (zinc chlorid double salt). The test is made by adding to 10 c.c. of milk 2 c.c. of the reagent, mixing well, sealing with melted paraffin, and holding at 37° C. in a water bath. By the test, according to Auzinger, good milk reduces the color in eight to twelve minutes, milk rich in bacteria reduces in five minutes or less, and when colostrum is present two or more hours are required.

Of the two reductase tests, according to Schardinger, reduction by the slow method is due to ferments produced by bacteria, while by the hastened method reduction is due to the natural ferments of milk.

STORCH TEST.

This test is made by adding to 5 c.c. of milk one drop of 0.2 per cent H_2O_2, and two drops of a 2 per cent solution of paraphenylendiamin and thoroughly mixing. The reagent must be freshly made at least every two weeks.

WILKINSON AND PETERS TEST.[1]

This test is made by adding to 10 c.c. of milk 2 c.c. of a 4 per cent alcoholic benzidine solution and two or three drops of acetic acid, then mixing well and adding 2 c.c. of 3 per cent H_2O_2.

GUIAC TEST.

The reagent is made by adding one part of guiac to ten parts of acetone. To make the test several drops of 0.2 per cent H_2O_2

[1] *Ztschr. Nahr. u. Genussm.*, 1908, 16, p. 172.

and 1 c.c. of the guiac solution are added to 10 c.c. of milk. The reaction appears in one to three minutes.

BELLEI TEST.[1]

The test is made by adding to 10 c.c. of milk three drops of 1.5 per cent aqueous solution of ortol and two drops of 3 per cent H_2O_2.

The Storch, Wilkinson and Peters, guiac, and Bellei tests are used primarily to detect heating above 70° C. and are of little value with us, as heating to such high temperatures is seldom resorted to in this country.

RESULTS OBTAINED

The results obtained with ferment tests are shown in Tables 34–37.

TABLE 34.

SHOWING AVERAGE, HIGHEST, AND LOWEST CATALASE DETERMINATIONS OBSERVED IN MILKS AT THE TIME OF DELIVERY, AND HIGHEST OBSERVATIONS MADE BEFORE COAGULATION OCCURRED AT THE VARIOUS TEMPERATURES OF STORAGE.

Supply	No.	As Received			After Storage			
		Average	Highest	Lowest	37° C.	Room Temp.	Ice-Box Temp.	1° C.
Certified	1	2.53	3.4	1.4	6.4	4.2	4.4	15
	7	2.52	4.2	0.8	3.0	7.4	15.0	20
	8	2.26	2.4	1.2	4.2	8.2	15.0	15
	9	2.0	2.6	1.2	4.2	7.8	15.0	15
	10	1.92	2.8	1.4	4.0	7.8	8.0	10
	11	1.63	2.0	1.2	3.8	2.0	5.8	15
	14	1.8	2.2	1.0	15.0	4.0	15.0	20
Insp.	4	3.06	4.0	2.4	3.2	4.4	3.6	20
	12	2.78	4.8	1.2	3.4	4.6	7.6	15
Pasteurized	2	1.23	1.6	0.2	4.4	4.4	15.0	20
	3	1.7	2.5	0.6	2.6	5.2	5.6	20
	13	1.7	2.5	0.8	2.8	4.6	10.4	15
	4½	0.98	1.4	0.6	1.0	1.6	5.4	2

TABLE 35.

SHOWING AVERAGE CATALASE DETERMINATIONS IN MILKS AS RECEIVED AND AVERAGE OF HIGHEST OBSERVATIONS BEFORE COAGULATION OCCURRED AT THE VARIOUS TEMPERATURES OF STORAGE.

	As Received			After Storage			
	Highest	Lowest	Average	37° C.	Room Temp.	Ice-Box Temp.	1° C.
Certified	4.2	0.8	2.1	6.51	5.91	14.03	18.5
Inspected	4.8	1.2	2.92	3.3	4.5	5.6	20.0
Pasteurized, 2, 3, 13	2.5	0.2	1.54	2.45	4.7	12.0	22.0

[1] *Centralbl. f. Bakt. u. Parasit.*, 1904, 12, p. 518.

Raw milks liberate more oxygen from hydrogen peroxid than do pasteurized milks. At the higher temperatures of storage catalases are not as much increased up to the time of coagulation as they are when milks coagulate at the lower temperatures. The tests are of value when milk ages at the temperatures usually met with in the milk trade and any reading above five should suggest further investigation.

TABLE 36.

SHOWING RESULTS OF REDUCTASE TESTS AND BACTERIAL COUNTS MADE ON THE MILKS WHEN RECEIVED.

Supply	No.	Slow Reductase (hours)		Hastened Reductase (minutes)			Bacteria per c.c.		
		Fastest	Slowest*	Average	Fastest	Slowest*	Average	Highest	Lowest
Certified	1	1:00	60%—	9.1	5	17	3,756	10,500	800
	7	...	100%—	8.3	7	19	6,650	27,000	1,800
	8	1:30	43%—	....	9	10%—	8,935	24,000	1,400
	9	....	100%—	....	9	30%—	20,650	51,000	3,500
	10	....	100%—	...	12	10%—	13,900	52,000	900
	11	....	100%—	10.7	7	22	3,543	14,000	600
	14	5:00	80%—	9.4	12	4	3,470	7,100	800
Insp.	4	2:00	40%—	10.1	8	13	33,610	52,000	12,500
	12	2:30	80%—	13.1	8	44	11,545	99,000	1,300
Pasteurized	2	4:30	50%—	....	9	60%—	310,250	840,000	6,000
	3	3:00	30%	....	110	80%—	285,750	1,640,000	16,000
	13	12:00	60%	....	25	40%—	91,600	1,569,000	4,000
	4½	....	100%	....	20	80%—	4,020	9,700	200

* The figures in these columns show the longest time taken to decolorize the specimens. The percentage figures show what percentage of specimens were negative or failed to decolorize.

Examination of Table 36 shows plainly that reductases must be of two kinds, those naturally present in milk and those produced by the bacteria. The slow reductase test is not entirely reliable for the determination of numbers of bacteria present, as is seen by comparing the observations on supplies 1 and 8 with supplies 9 and 10. All bacteria do not produce reductases which can be tested for by the Schmidt-Müller test, for pasteurized milks at times showed high bacterial counts and still such milk did not reduce at all or only slowly. When milk is coagulated the tests are no longer satisfactory.

These tests were tried on milks that had been stored at the different temperatures. Generally with the increased bacterial count there was an *accelerated* reduction with the Schmidt-Müller test, and while this test is not absolute, decolorization in less than

one hour is practically always an evidence of a high bacterial content, and decolorization in less than two hours should indicate further investigation.

The hastened or Schardinger reductase test is of great value, not as an indicator of bacterial content, but for the detection of heating of milk. Milk heated to 140° F. or above, while exposed to the air, generally will not reduce by this test, and when milk pasteurized in bulk reduces it is evidence of inefficient pasteurization. Likewise *mixed* milk that will not reduce has been heated

TABLE 37.

SHOWING CONCENTRATION OF FERMENTS IN GRAVITY CREAM, WHEN MILKS WERE STORED AT 1° C. FOR 14 DAYS.

Supply	No.	Catalase			Hastened Reductase (minutes)			Slow Reductase (minutes)		
		Cream	Skimmed	Mixed	Cream	Skimmed	Mixed	Cream	Skimmed	Mixed
Certified	1	40.0	3.0	6.0	1	9	5	3	Neg.	4
	7	50.0	15.0	20.0	1	4	2	7	20	10
	9	60.0	1.8	11.2	2	15	5	3	Neg.	70
	10	50.0	4.2	30.0	3	15	6	4	318	30
	11	15.0	1.2	2.4	1	24	11	2	120	2
	14	20.0	1.8	9.2	5	Neg.	20	1	7	4
Insp.	4	15.0	3.2	5.6	20	40	30	5	10	4
	12	60.0	3.8	5.2	2	15	5	[illegible]	Neg.	11
Pasteurized	2	40.0	1.6	2.0	3	6	4	6	19	16
	3	16.2	4.0	3.0	20	40	30	Coag.	Coag.	Coag.
	13	40.0	4.0	5.2	10	40	20	8	Neg.	20
	4½	6.0	0.0	0.8	3	Neg.	Neg.	30	Neg.	Neg.

to 140° F. or above. Here, however, it must be remembered that milk from one cow may not contain reductases detectable by this test. In two different herds a number of cows were found whose milk did not reduce. Heating in the sealed bottle does not destroy reductases as markedly as does heating when milk is exposed to the air.

Storch, Wilkinson and Peters, guiac, and Bellei tests were applied throughout the entire year. These tests are of little value unless milk has been heated beyond 70° C. Only a few bottles of pasteurized milk showed overheating during the entire time milks were received at the laboratory.

Babcock and Russell (*loc. cit.*) in 1897 in connection with experiments on the ripening of skimmed milk and full cream cheeses pointed out that in any creaming process the natural enzymes of milk are separated largely from the skimmed milk. In my investi-

gation on gravity cream and skimmed milk the appearance of ferments in gravity cream was noted. Table 37 shows some of the results obtained.

This table shows that gravity cream contains larger amounts or more active ferments than does the skimmed milk. Even in pasteurized milk the ferments in cream are considerable. Whether separator cream contains relatively more ferments than the milk from which it is obtained I have not determined. Babcock and Russell's experiments show that much galactase is lost in separator slime. The importance of ferment concentration in gravity cream may be considerable in infant feeding.

VIII. SUMMARY AND CONCLUSIONS.

Milk from the better class of dairies in large cities is generally delivered in neat and clean bottles. Good milk and good service are so important to the consumers that the drivers of milk wagons soon become interested in furnishing good milk to their family trade.

Tests that give results readily and quickly are most important because a new supply of milk must be available daily. It is of little value to the consumer to learn that the milk he used yesterday or a week ago was unfit.

Our better classes of milk contain little sediment, market milks containing more dirt but fewer cells than certified milks. The Tromsdorff tube is a convenient method for examining for dirt and cells.

It is relatively easy to produce milk of low bacterial content but the greatest care must be constantly exercised to prevent occasional high counts.

Increase in bacterial content was more rapid at the higher temperatures, and at a temperature just above freezing there was little bacterial multiplication until after the seventh day, but from that time on the increase was rapid. Cream on rising carries with it a large portion of the bacteria in milk. Separator cream does not take with it as large a portion of the bacteria as does gravity cream. When milk was stored and the cream and skimmed milk were not remixed, bacterial multiplication was not as great during the same period of storage as when the milk was mixed repeatedly.

Of the bacteria in good milks about 30 per cent were acid-producers, while 20 per cent of those in inspected and 30 per cent of those in certified and pasteurized milks were protein-digesters. At the higher temperatures fermenting organisms increased more rapidly in certified milk, but at the lower temperatures the most marked increase was observed in pasteurized milk. The percentage of peptonizing types increased only at low temperatures. Apparently gravity cream contains the larger portion of peptonizing forms, while the skimmed milk below contains most of the fermenting forms when milk has been refrigerated.

In a lactose medium 41.43 per cent of certified, 55 per cent of inspected, and 84 per cent of pasteurized milks produced gas. Certified milk did not contain bacteria producing hydrogen sulfid and indol as frequently as did inspected and pasteurized market milks.

Milk from definitely diseased quarters is less acid than from healthy quarters. At the time of delivery the acidity of milks varied markedly, the average being lowest for pasteurized milks and highest for inspected milk. The acidity in all milks increased more markedly at the higher temperatures of storage, and at all temperatures the increase was greatest in pasteurized milk.

Coagulation occurred as soon after delivery in pasteurized market milk as in raw milks. Milk stored at a temperature just above the freezing point showed marked bacterial increase before coagulation occurred. The acidity of milk at which coagulation occurred varied. It was highest after storage at 37° C. and room temperature, and lowest when milk was stored in the ice-box. Certified milk coagulated at a lower acidity than did the other grades of milk but it took longer to get the coagulating acidity in certified milk. Alkaline milk was very infrequent.

When clotting had occurred the curd was most acid. The straw-colored fluid under the cream is an evidence of peptonization and was not as acid in reaction as the curd and whey. Gravity cream, above the skimmed milk, was as acid as the skimmed milk as long as the acidity of the mixed milk was not high.

On long storage butyric acid and putrefactive odors became marked. When milk was stored at low temperatures the develop-

ment of putrefactive odors may not have been preceded by acid odors. If milk is stored for a long time the protein of certified and pasteurized milks may be entirely broken down.

Protein decomposition without coagulation occurred principally at a temperature near the freezing point.

Adding an equal part of 68 per cent alcohol to milk is an easy and reliable test for the detection of beginning acidification.

The tests for catalases and reductases are of much value. The catalase and slow reductase tests are of assistance in detecting old milk, and the hastened reductase test offers a convenient and reliable method for detecting and testing the efficiency of pasteurization. Gravity cream carries with it a large portion of the ferments of milk.

The fear of putrefying organisms in pasteurized milk is not warranted as far as market milk pasteurized by the holding method is concerned. While predominance of putrefying bacteria is not the only objection to pasteurized milk, it has been an important one. Certified milk, because it contains but little cow manure, is infected principally with spore-bearing organisms, it is always well refrigerated, and contains as large a percentage of protein-digesting and no more acid-forming bacteria than does pasteurized milk.

The cleanliness necessary to produce certified milk is so imperative and the variation in the production and pasteurization of ordinary market milk so marked, that certified and pasteurized milks are most variable. We find therefore such great variations in numbers and kinds of bacteria in certified and pasteurized market milk that the bacterial content cannot be foretold, the best information being that based on a large number of observations of a number of supplies of these grades of milk. Inspected milk is apparently more uniform in numbers and kinds of bacteria.

These investigations were made possible by the establishment of a milk research fellowship in the Department of Preventive Medicine and Hygiene of the Harvard Medical School by the Milk and Baby Hygiene Association of Boston. I wish here to acknowledge my appreciation of the interest and valuable suggestions and assistance of Professor M. J. Rosenau, as well as to express my thanks to the Milk and Baby Hygiene Association for giving me the opportunity to make these investigations and to the various producers and distributers of milk in Boston and vicinity who so kindly supplied me with the milk used.

A STUDY OF STREPTOCOCCI FROM MILK AND FROM EPIDEMIC SORE THROAT, AND THE EFFECT OF MILK ON STREPTOCOCCI.*

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During the recent epidemic of sore throat in Chicago, shown by Capps and Miller[1] to have been spread by milk, I had the opportunity to study streptococci from numerous throats, from the pus of suppurating glands, and from exudates in a number of fatal cases. In a joint note with Dr. D. J. Davis[2] it was pointed out that these streptococci possessed certain peculiarities: They appeared mostly as diplococci and short chains surrounded by a capsule; their growth on blood agar was not mucoid, but more abundant and with less hemolysis than that of the typical streptococcus pyogenes, from which they, however, did not differ in fermentative properties. In no instance was inulin fermented. For a more complete description see the paper by Dr. Davis and myself and the recent paper by Davis.[3] It was found that these properties varied according to the severity of the infection. Many cases of ordinary tonsilitis yielded the ordinary streptococcus pyogenes. The special features mentioned were most marked in fatal cases, and the strains isolated from the peritoneal exudate and blood would show them to a greater degree than the ones isolated earlier in the attack or at the same time from the tonsils. After cultivation on blood agar it was noted that the strains from the tonsils soon lost any distinctive peculiarities, whereas those from the exudates in the fatal cases retained them longer. This was true not only of the strains from different cases but also of the strains from the same case. Thus, in four instances the throat strains lost the special properties in from one to four weeks, whereas two of the strains from the exudates were still growing in the original manner, though not so marked, after six and seven months. Hence it is clear that the cases were due to streptococci with distinctive morphological and

* Received for publication September 1, 1912.

[1] *Jour. Am. Med. Assoc.*, 1912, 58, p. 1848. [2] *Ibid.*, p. 773. [3] *Ibid.*, p. 1852.

cultural features, and, furthermore, that as a result of growth in the fluids of the body these features may become accentuated and more permanent.

The epidemics in Boston[1] and in Baltimore[2] were of the same general nature as the one in Chicago, being milk-borne, and associated with similar streptococci. It should be noted that each epidemic occurred during the winter or early spring, seasons when sore throats and tonsilitis are common. In other milk-borne epidemics, in which blood agar methods were not used in the study of the streptococci, it is impossible to say whether the streptococci resembled the ones described by us.

The source of the streptococci in milk-borne epidemics of sore throat, whether from diseased udders or from the throats of milkers or others handling the milk, has been discussed a great deal. Davis[3] points out that during the epidemic in Chicago there existed an unusually large number of cases of mastitis in the cows which furnished the incriminated milk, and that tonsilitis among the milkers was also common. Most observers now hold that the streptococci found in milk cannot be differentiated clearly, culturally or morphologically, from the ordinary streptococcus pyogenes. Puppel[4] and Ruediger,[5] however, believe that this may be done by means of blood agar plates, as the milk streptococci do not cause hemolysis while the streptococcus pyogenes does. Gminder[6] has shown that streptococci from chronic mastitis in cows are of little virulence for rodents but produce mastitis in goats. Puppel is also inclined not to attribute much importance to the streptococci in mastitis, and Heinemann,[7] by animal passage, succeeded in making the milk streptococcus as virulent as the streptococcus pyogenes. Notwithstanding the recent evidence that milk not infrequently spreads streptococcus sore throat, the attempts to isolate virulent streptococci from milk have usually resulted in failures. I shall now present certain results that I have obtained from the study of milk in relation to streptococci.

[1] Winslow, *Jour. Infect. Dis.*, 1912, 10, p. 73.

[2] Hamburger, *Jour. Am. Med. Assoc.*, 1912, 58, p. 1109.

[3] *Loc. cit.*

[4] *Ztschr. f. Hyg. u. Infectionskr.*, 1912, 70, p. 3.

[5] *Science*, 1912, 25, p. 223.

[6] *Centralbl. f. Bakt.*, I, Orig., 1912, 63, p. 152.

[7] *Jour. Infect. Dis.*, 1907, 4, p. 89.

THE ISOLATION OF VIRULENT STREPTOCOCCI FROM "SEPARATOR SLIME" AND FROM MILK, CREAM, AND BUTTER.

In my work on experimental endocarditis I learned how harmless non-virulent bacteria are when injected intravenously in rabbits and guinea-pigs, and how rapidly their destruction takes place. It was thought that this method might serve to separate avirulent from virulent streptococci and other bacteria in milk.

There is being introduced into the dairy industry by the De Laval Separator Company a clarifying machine. The "slime" obtained in this way and also from the bowl of the ordinary cream separator contains enormous numbers of streptococci and other bacteria, leukocytes, and foreign matter. This material seemed specially adapted for use in the tests to be made. The "slime" was collected in sterile bottles, packed in ice, and sent to the laboratory. Rather dense emulsions were made in NaCl solution, centrifugated fractionally, and the turbid supernatant fluid examined in various ways. Nine samples of "clarifier slime" and six samples of "separator slime" were examined. The former represented four central stations, the latter six individual dairies, some of the latter being separated by a distance of 400 miles. The samples at the time examined were from 24 to 72 hours old and all reacted acid to litmus. Rabbits were injected with from 6 to 15 c.c. of the suspension depending upon the size, guinea-pigs with from 5 to 7 c.c., while mice received from 0.5 to 1 c.c. In order to make sure that the results were not due in part to other material in the milk than bacteria, cultures were made in ascites meat broth, incubated for 12 to 18 hours, and then injected. Both methods gave the same results. The animals usually died of streptococcemia in from 24 to 72 hours. Smears of the emulsions, cultures on blood agar plates, and broth cultures showed streptococci in greatly predominating numbers in all the samples. The next most common organism was what appeared as a pseudo-diphtheria bacillus. In order to test whether the strains isolated from the blood of the animals which died were really pathogenic, a second and in some instances a third inoculation of small size was made. Virulent streptococci were obtained after death of the animals from the blood or peritoneal exudate or from both in 13 of the 15 samples

injected. Intravenous injections in the rabbits gave the highest percentage of positive results. In most cases (24) streptococci in pure culture were obtained, streptococci in predominating numbers but mixed with colon bacillus in nine, and the colon bacillus pure in one. One animal yielded a pure culture of a typical pneumococcus. Nine animals survived while three developed streptococcus arthritis later. It should be stated that in the six dairies from which "slime" was obtained there were no cases of mastitis in the cows and no tonsilitis in the milkers, at least so far as was known. In the case of the central stations, these points could not be ascertained definitely. The blood agar plates showed hemolyzing colonies of streptococci in small numbers in only four samples; non-hemolyzing streptococci were obtained in large numbers in all. From one sample the plates yielded an organism which resembled morphologically and culturally (growing in symbiosis with streptococci) the influenza bacillus, but it was not found after death in the animals injected with this sample.

Virulent streptococci were obtained also from two samples each of milk and cream, obtained in the open market and pasteurized by the flash method. One of two samples of butter gave a similar result. The blood agar plates again showed the non-hemolyzing streptococci in large numbers, but no hemolyzing colonies. Milk pasteurized by the holding process yielded negative results.

The difference in the character of the growth of the streptococci on blood agar plates before and after animal passage was striking, hemolyzing colonies only being present on the plates from the blood of the animals and almost exclusively non-hemolyzing colonies on the plates made directly from the suspensions. This finding brings up the point whether the non-hemolyzing streptococci become hemolytic on animal passage or whether the virulent streptococci were present in such small numbers as to be missed in the first plates. The tests for virulence before animal passage of strains which came from single non-hemolyzing colonies and a number of hemolyzing colonies resulted negatively even though very large doses were injected. In view of this fact, and because of the total absence of non-hemolyzing colonies in the animals after death, it would seem that virulent streptococci were present in small num-

bers and hence missed in the plates from the original material. This seems to explain why virulent streptococci are obtained from milk so rarely by the plate method. Most of the strains of streptococci obtained from the animals appeared in diplococcus forms and as short chains, surrounded by a well defined capsule. Hemolysis was less marked about those colonies which were particularly large and moist and consisted of cocci with an abundance of capsular substance. These features were soon lost on artificial cultivation, the organisms now resembling the ordinary hemolytic streptococcus. One strain was passed through three successive guinea-pigs. It now grew exactly like streptococcus mucosus, had a wide capsule, and did not ferment inulin. This strain in particular as well as one other resembled very closely indeed those obtained from the throats during the epidemic of sore throat. This was believed not to be a mere coincidence, and it was decided to test the effect on streptococci of milk obtained in as sterile a manner as possible.

EXPERIMENTS WITH MILK OBTAINED IN STERILE FORM.

The following technic was used in an attempt to procure sterile milk directly from the cow. Milking tubes, well known in the dairy industry, were sterilized after being provided with a metal hood and a suitable container. The attempt was made in a model dairy where rigid cleanliness is enforced. The udder was washed with a solution of formalin; the teat to be used was milked approximately one-fourth empty and the mouth of the teat sterilized with 1-1,000 bichloride; the tube was inserted, a rather large amount of the milk allowed to escape, and then 200 c.c. of milk were collected under a hood in each of two bottles. Part of this was tubed at once, from 0.5 to 4 c.c. being placed in each tube. Plate cultures on plain and blood agar were made at once from the bottles to determine the number of bacteria present. Part of the samples were pasteurized (at 60° C. for 20 min.), part placed in the incubator, and part in the ice-chest. Five of 12 normal cows yielded sterile milk in this way. The number of colonies in the milk of the others per c.c. was as follows: 800, 4,000, 3,500, 400, 4, 14, and 9. Three of these showed streptococci in large numbers after the milk was incubated, and two of these when injected into

guinea-pigs proved virulent. The milk of one cow yielded a typical virulent pneumococcus. These three cows had not had any disease of the udder at any time. (The milk from two cows with chronic mastitis contained an enormous number of hemolyzing streptococci in pure culture, one loop yielding countless numbers of hemolyzing colonies on blood agar. Both proved only moderately virulent for rabbits, guinea-pigs, and white mice.)

The effect of the sterile milk on streptococci was now tested. First aerobic and anaerobic cultures on blood agar were made to determine whether the milk to be used was sterile. The tests were in duplicate. After inoculation with ordinary streptococci one set of tubes was placed at 37° C., the other kept at room temperature and part of the time in the ice-chest, thus imitating the conditions that exist in the routine handling of milk. It soon became apparent that the tubes placed at 37° C. and in which growth, with acid production, occurred did not appreciably change the streptococci, whereas the streptococci in the milk kept outside the incubator were modified perceptibly. Streaks with this milk on the surface of blood agar plates showed a more abundant growth and less hemolysis than did the streaks from the incubated milk. That the milk carried over with the loop was not the cause of this difference was shown by placing a loopful of culture from the surface of blood agar slants into milk and making the streaks at once. No change occurred.

The effect of whole milk was studied on 15 strains of virulent streptococci. Four of these strains were ordinary hemolytic streptococci. Five—all growing like typical hemolytic streptococci—came from the animals in the above experiments and six from cases of sore throat during the epidemic. Four of the latter had reverted to typical streptococci while two still grew in the peculiar manner. The strains were obtained originally from single colonies, but were plated out a second time and subcultures again made from a single colony in order so far as was possible to be sure that the results obtained might not be due to a mixture of strains.

The experiments with the following strain (595) illustrate in general the results obtained. This strain had been isolated from the throat of a case of scarlet fever nine months previously and had

since been cultivated continuously on blood agar. During this time it had no capsule and always produced small colonies surrounded by a wide zone of hemolysis. On May 9 a subculture was made on blood agar; on May 11 a culture was made from this on a blood agar slant and also two inoculations of sterile milk. The slant and one of the tubes of inoculated milk were placed at 37° C; the other tube of milk was kept at room temperature and in the ice-chest. On May 14 subcultures were made side by side from each of the tubes on blood agar plates, which were placed at 37° C. The plates from the blood agar slant and from the incubated milk tube (which had become strongly acid in reaction) gave only scant growth but with marked hemolysis; no capsules could be demonstrated in either case. The growth from the milk tube kept at a low temperature was more abundant. At the end of 15 hours there was no trace of hemolysis, at the end of 24 hours a rim one millimeter wide had formed which at the end of 48 hours was about two millimeters in width. The cocci were surrounded by a definite, eosin staining capsule. Subcultures on blood agar soon lost these features even though the inoculations were made with streptococci placed in a loop of milk just previously. On the other hand, alternate cultures for 24 hours on blood agar and in milk soon increased the capsular substance and reduced the hemolysis.

It was now determined to test the virulence of the two types. Both were inoculated into ascites broth and grown for 24 hours. The culture from blood agar showed rather long chains with no capsule, while those from the third generation in milk consisted chiefly of diplococci with only an occasional short chain, surrounded by a capsule. Diminishing but equivalent doses of each strain were injected intraperitoneally in mice, and all died at the end of 72 hours. Those which had received the milk-treated cocci died first, and their blood contained encapsulated cocci, while the blood of the mice injected with cocci from blood agar showed typical hemolytic cocci. Two guinea-pigs were injected subcutaneously with the blood agar strain, and survived without developing symptoms; those injected in the same way with the milk-treated strain developed a marked local reaction and arthritis, and died in 12 days. Cultures from the indurated area and from the blood yielded a pure culture of a streptococcus which resembled

those from the fatal cases in man; the joint cultures remained sterile.

Some of the throat strains, recently isolated, were modified even more strikingly by this treatment, whereas a highly virulent strain from the blood of a fatal case of bronchopneumonia in man was not influenced perceptibly by the milk.

Treatment with milk seemed to have a marked effect on strains from the fatal cases during the epidemic which had lost part of their original peculiarities. A single soaking in milk would bring back fully the former characteristics. This was true also of two strains from the Boston epidemic, now 18 months ago, kindly given me by Dr. D. J. Davis.

One of my strains, cultivated for six months, reverted to the original type on passing growths on blood agar through two guinea-pigs; similar passage of a strain grown in milk resulted in growths exactly resembling the streptococcus mucosus.

In this connection it should be mentioned that subcultures on blood agar plates from the upper and much-dried portion of old blood agar tube-cultures (72 days) of streptococci from fatal cases for several generations produced dry fine colonies surrounded by a well defined zone of hemolysis, while cultures from the lower and moist part of the same old tubes gave moist large colonies with little hemolysis. Concentration of salts during the evaporation of the agar would seem then to have an influence on the morphology and amount of hemolysis produced by the streptococci. This point should be studied further.

As unheated sterile milk has the most pronounced effect on streptococci, pasteurized milk a somewhat less pronounced effect, and autoclaved milk little or no effect, salt concentration cannot be the factor here. The effect of the milk is independent of the cream content. It is interesting that the modification should occur only when growth of streptococci is prevented or delayed by placing the mixtures at a low temperature.

A comparative study of the resistance to heat of various streptococcal strains has also been made. Suspensions from blood agar slants were made in broth and unheated sterile milk. These were then heated to 45° and 60° C. for 20 minutes respectively. The lower temperature of 45° C. was sufficient to sterilize the strains of

high virulence in the milk suspensions, whereas none were sterile of those suspended in broth. Most strains from blood agar plates, which were non-virulent, showed growth after heating to 60° C. for 20 minutes, whereas the virulent strains similarly treated all were killed.

SUMMARY.

Streptococci, virulent for animals, but which differ from typical streptococcus pyogenes in a more abundant growth, in being encapsulated and not forming chains, and in causing but little hemolysis, occur in predominating numbers in epidemic sore throat of milk-borne origin. On artificial cultivation these strains sooner or later assume the characteristics of streptococcus pyogenes.

Cultures on blood agar plates from ordinary milk usually give rise to colonies of streptococci that do not cause any hemolysis, but the injection of rabbits and other animals with milk "slime" practically always produces infection with encapsulated, but otherwise typical, hemolytic streptococci. The blood agar plate method consequently is not a reliable means with which to search for streptococcus pyogenes in milk.

By placing streptococcus pyogenes in unheated milk it becomes modified so as to correspond to the streptococci in epidemic sore throat. The modifications may be accentuated by passage through guinea-pigs, and in some cases cultures like those of streptococcus mucosus may result.

The fact that milk so modifies streptococci is an additional indication of the important part it may play in epidemic sore throat. It is not possible to determine whether the streptococci in such epidemics are of exclusively bovine or human origin; they may be of both.

Milk drawn in a sterile way from normal cows may contain virulent streptococci and pneumococci; hence "certified milk," while surely less contaminated than ordinary milk, may contain pathogenic bacteria, and the advisability of pasteurization even in this case should be considered, especially during seasons when sore throat is common.

Butter and cream may contain virulent streptococci.

THE CONTENT IN ANTIBODIES OF NORMAL HUMAN COLOSTRUM AND MILK.*

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Famulener,[1] in an extensive study of the transmission of immunity from mother to offspring, found that goats actively immunized against sheep blood transmit the specific hemolysin to their young, and that the colostrum is the chief agent of transmission, the placenta playing only a minor rôle. He found that the colostrum carries a high content of specific hemolysin when immunization is effected during the period of gestation, the antibodies rapidly disappearing from the milk after the mother is suckled by the young.

On account of this difference in the amount of hemolysin in the colostrum and milk of immunized goats, some observations were made to determine whether the same difference occurs in the antibody content of normal human colostrum and milk. For this purpose the opsonins for the streptococcus, the staphylococcus, and the tubercle bacillus were estimated. Three specimens of milk were examined, two taken on the fifth and the third on the seventh day after delivery. Three specimens of colostrum were studied, two taken before, one after, delivery. The women were all normal. The milk and colostrum were collected in sterile tubes, centrifuged for about an hour, and the lower fluid portion removed from under the cream layer. Part of this was kept at 56° C. for one-half hour and then examined for its opsonic content, while the other part was examined unheated in the usual way. The unheated milk and colostrum were also diluted to the point of opsonic extinction and their opsonic content measured by this method. The unheated blood serum of two of the women was also examined for its opsonin. In the heated specimens the milk contained no opsonin while the colostrum contained an appreciable amount. For

* Received for publication September 1, 1912.

[1] *Jour. Infect. Dis.*, 1912, 10, p. 332.

example, with practically no bacteria ingested in the presence of milk, 0.2 streptococci per leukocyte were taken up in the colostrum specimen. The same was true of the staphylococcus and tubercle bacillus. One specimen of unheated milk contained no opsonin for the streptococcus, another contained a trace, while the unheated colostrum contained at least twice as much opsonin as this milk. By diluting the unheated milk and the colostrum to the point of opsonic extinction, the point of extinction for the milk with respect to the streptococcus was 1:12, while that for the colostrum was 1:48.

The unheated blood serum was found to contain considerably more opsonin than the milk or colostrum. For example, one serum had a streptococcus phagocytic index of 0.8, 32 per cent of the cells being phagocytic, while the index of the unheated colostrum was 0.18, 8 per cent of the cells taking part in phagocytosis. Another woman with a phagocytic index of 0.5 for the serum, 48 per cent of the leukocytes being phagocytic, had no streptococci ingested in the presence of the unheated milk.

From these experiments one may conclude that normal human colostrum-milk contains more opsonins for the streptococcus, staphylococcus, and tubercle bacillus than does the later milk, but less than the blood serum. Since this is true, it would seem of great importance, as Famulener points out, that the newly born infant should receive the colostrum-milk.

STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS.*

I. THE PERMEABILITY OF TUBERCLES FOR IODIN COMPOUNDS AND PROTEINS.

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GENERAL CONSIDERATIONS.

The principles of chemotherapy, as laid down by Ehrlich, are of so fundamental a character that there is no limit to their application in infectious diseases, and possibly in other conditions, notably cancer. With the spirilloses and trypanosome infections, in which most of the work has so far been done, the conditions are favorable for the meeting of the drug and the germ, since with most forms of these diseases the germ lives chiefly or entirely in the circulating fluids. It is noteworthy that the only disease in which "Therapia magna sterilisans" has been practiced successfully on an empirical basis is also a blood infection, malaria. The consideration of tuberculosis from the standpoint of chemotherapy brings in distinctly new problems owing to the fact that the bacteria are, in large part, located at points specifically removed from the circulation by proliferating tissues. The avascularity of the tubercle must of necessity have a large influence on the meeting of the drug and the germ, and this condition has perhaps been responsible for the lack of success of the innumerable empirical attempts at chemotherapy which have been made with the disease in the past. Avascularity of an infected tissue may, perhaps, make for either assistance or hindrance in chemotherapy, for we can imagine that the drug might accumulate in the avascular area, just as, for instance, calcium salts do, or, entering avascular and vascular tissues alike, it might remain longer where there is no circulation. Absence of living cells may also make a difference in that certain drugs may be either destroyed or activated by living cells, and hence have either

* Received for publication August 24, 1912.

a greater or a less effect in the necrotic portions of the tubercle than elsewhere in the body. These and other points present themselves, and to attack the problem of tuberculosis chemotherapy it would seem to be necessary to learn first to just what extent different classes of chemical substances enter tubercles, both early and advanced, how much they tend to accumulate specifically in the tissues, and how long they remain there. For a chemical which is to destroy the tubercle bacillus, it would seem, should be one that will enter readily into the avascular tuberculous lesions, and, if possible, enter or accumulate in such tissues more than in normal tissues.

The problem is further complicated by the chemical composition of the tubercle bacillus itself, with its large proportion of resistant fatty and waxy material, which must, it would seem, make its permeation and destruction a very different matter from the attack upon the naked and delicate trypanosomes, spirillae, and spirochaetes. Hence the permeability of the tubercle bacillus for chemicals of different classes becomes a fundamental question in connection with the main problem. In the investigation of the subject the fatty matter of the tubercle bacillus, while perhaps an obstacle to chemotherapy, makes attack of the problem appear somewhat easier, since the permeability of the bacteria must be largely determined by this substance which can be extracted from them in large amounts and rendered available for experimental work *in vitro*, without, at the beginning, calling for the extensive animal experimentation which is essential in the study of the chemotherapy of protozoan infections. The influence of the fatty constituents of the cells upon the permeability of tissue cells to drugs and dyes has already been extensively investigated, and we have, therefore, many clues for investigation of the permeability of *B. tuberculosis*.

In planning a systematic investigation on the chemotherapy of tuberculosis, therefore, it seemed desirable first to determine the entrance of various classes of substances into the tubercle and into the bacteria, since the effective tuberculocide must be, theoretically, one which enters freely and, if possible, selectively into the avascular tubercle, and with like facility passes through the fatty

sheath of the bacillus. We have found it possible to attack directly some of the problems involved, while others have called for preliminary studies of certain fundamental questions. Some of the work has advanced sufficiently to warrant a preliminary report, which should be introduced by stating that all the work reported in this and in subsequent articles has been done through the co-operation of several persons, each of whom has helped in various stages so that it is difficult to credit any particular step to any one or two persons. Those engaged in various aspects of the work here reported are Dr. Lydia DeWitt, Dr. H. J. Corper, Dr. G. L. Kite, Miss Hope Sherman, Mr. G. C. Lake, and ourselves.

ENTRANCE OF SUBSTANCES INTO TUBERCLES AND OTHER LESIONS.

HISTORICAL.

The only study we can find directly concerning itself with this topic is that of Oswald Loeb and Michaud.[1] A study of the distribution of iodin in normal animals had been made previously by Loeb,[2] who found that when injected in the form of KI, the brain, spinal cord, bone marrow, and fat tissue were usually free from iodin, the muscle contained very little, and then, in increasing amounts, the liver, lymph glands, kidneys, salivary glands, lungs, blood, and, of course highest of all, the thyroid. When compounds of iodin which are soluble in fat were injected (iodoform, ethyl iodid) it was found in the brain, spinal cord, and fat tissue. After ethyl iodid injection he found the iodin especially in the lungs, where it is excreted, while more iodin is found in the kidneys and salivary glands after KI injection, for the same reason.

When these same iodin compounds were injected into tuberculous rabbits and guinea-pigs, Loeb and Michaud found that regularly the tuberculous tissues took up a disproportionately large amount of iodin. Thus, four rabbits inoculated in one eye with tuberculosis showed from one and one-half to two and three-fourths times as much iodin in the tuberculous eyes as in the normal eyes, and tuberculous lungs were found to contain increasing amounts of iodin in proportion to the amount of tuberculous tissue they contained. Caseous lymph glands of guinea-pigs contained more iodin than any of the normal organs.

This important investigation has only recently begun to receive the attention it has deserved, and has as yet been neither confirmed nor extended so far as tuberculous lesions are concerned, although in view of the fact that these weighty conclusions rest upon a series of four rabbits and four guinea-pigs, and that the relatively inaccurate method of Baumann was used for the iodin determination, amplification is certainly required before entire acceptance is warranted.

Collateral support is given by two observations on cancer. Van den Velden[3] reported the case of a man who died of gall duct cancer, with secondaries in the liver

[1] *Biochem. Ztschr.*, 1907, 3, p. 301.

[2] *Arch. exp. Path. u. Pharm.*, 1907, 56, p. 321.

[3] *Biochem. Ztschr.*, 1908, 9, p. 54.

and pancreas, five and one-half hours after a subcutaneous injection of 3.0 gm. of NaI. Analysis showed iodin in abdominal, pleural, and pericardial fluids, and in two large secondary growths in the liver and pancreas, but none in the normal liver and pancreas tissue, despite the relatively avascular nature of the tumors. The absence of iodin in the normal tissues under these conditions is difficult to understand, and, in the light of our observations on animals, incredible. Takemura,[1] who found that iodin distributes itself in normal rats and mice much as Loeb found in guinea-pigs, (noting an especially high content in the skin), observed that in mice with cancer there is nearly as much iodin in the tumor tissue after injection of KI as in the tissues which normally contain the greatest amount of iodin; in sarcoma in rats the iodin in the tumor tissue was intermediate between iodin-rich and iodin-poor tissues. Our own experiments, as given below, indicate that these results may depend upon the amount of necrosis in the carcinomas and sarcomas. More recently Loeb[2] has reported the finding of a larger proportion of iodin in the enlarged glands removed by operation from a syphilitic (0.28–0.53 mg. per gm.) than in the blood of the same patient (0.082–0.088 mg.) 20 hours after the last dose of iodides.

In this connection might be mentioned the solitary observation of Loeb that the pus in a turpentine abscess in a rabbit injected with KI contained a larger proportion of iodin than the blood itself. Also, the now classical observation of Bondi and Jacoby[3] that injection of rabbits causes more of the injected salicylic acid to localize in joints, even when there is no arthritis; and the earlier observation by Fillipi and Nesti[4] that after aspirin has been given by mouth to persons with arthritis, the synovial fluid contains more salicylic acid than the urine. Other related observations are the following:

Blumenthal[5] observed that the addition of iodin to the atoxyl molecule causes it to enter into inoculable sarcomas of dogs and rats with special avidity, although even in the uniodized state the atoxyl is found more abundantly in these tissues than in the normal tissues. The effect of the atoxyl is to cause an increased rate of growth in the tumors of these animals.

Kapsenberg[6] states that an extract of tubercle bacilli made with water in the presence of chloroform, has a decided affinity for iodin, and that the resulting compound is specifically bactericidal for tubercle bacilli, but the data presented in this article are not sufficient to carry conviction.

Morel and Dalous[7] injected tuberculous guinea-pigs with anthrax cultures and found that the anthrax bacilli do not enter the larger tubercles; but in small tubercles consisting only of a giant cell and a single row of epithelioid cells, the protoplasm of each of these may contain anthrax bacilli.

EXPERIMENTAL.

We have undertaken to repeat the experiments of Loeb and Michaud, and to amplify them. Our plan of procedure was as follows: Guinea-pigs, the largest obtainable, were injected subcutaneously with human tubercle bacilli (0.01 mg. usually), and the animals were selected, as far as possible, when they had the maximum enlargement of the regional lymph glands before ulceration led to evacuation of their contents. In

[1] *Ztschr. physiol. Chem.*, 1911, 72, p. 78.
[2] *Arch. exp. Path. u. Pharm.*, 1912, 69, p. 108.
[3] *Hofmeister's Beitr.*, 1906, 7, p. 514.
[4] *Allg. med. Zentralztg.*, 1902, 71, p. 613.
[5] *Deutsch. med. Wchnschr.*, 1910, 36, p. 2275.
[6] *Berl. klin. Wchnschr.*, 1912, 49, p. 879.
[7] *Compt. rend. Soc. de Biol.*, 1907, 62, p. 74.

order to secure local lesions which could be compared with corresponding normal tissues, several large males were inoculated in one testicle, but this did not give useful results, for invariably the other testicle developed extensive tuberculosis. Equally unsuccessful was the result of direct intra-hepatic inoculations, which caused only an extensive local miliary tuberculosis which rapidly became generalized. Good results were obtained by inoculating human tubercle bacilli into the vitreous of one eye, and then, just before the eyeball was ready to rupture, injecting the iodin compound and analyzing separately the normal and the tuberculous eye.

In order to learn whether the entrance of iodin compounds into tubercles depends upon some peculiarity of the tubercles themselves, or whether it is common to necrotic areas and exudates in general, a series of experiments was performed as follows: Necrosis of the entire left kidney was produced in rabbits by aseptic ligation of the artery, vein, and ureter. This is followed by a severe engorgement of the organ from the collateral circulation through the capsule, which results in stasis and total necrosis of everything but the capsule. Necrosis of muscle was produced by injecting 2 c.c. of 50 per cent formalin (equal to 20 per cent formaldehyde) into the muscle of one thigh; this also produces a severe local subcutaneous edema from which sufficient fluid could usually be expressed to permit of analysis. Exudates were produced by injecting into the left pleural cavity one to two gms. of aleuronat suspended in 5–10 c.c. water, sometimes with one c.c. of turpentine or a loopful of solid *Staphylococcus pyogenes aureus* culture added to produce more violent reactions. To secure an inert colloid mass to compare with the dead tissues, subcutaneous injections were made of sterile five per cent agar jelly, 8–20 c.c. being injected at 50° C. by means of a powerful syringe such as is used for cosmetic work with paraffin. Analysis of this agar showed it to contain a negligible amount of iodin, about 0.001 mg. per 20 c.c. in the amounts used. Several or all of these different procedures were carried out in the same animal in most instances, thus permitting a comparison of the iodin determinations in several different lesions as well as with the normal tissues of the same animal.

The injections were made subcutaneously with the following iodin compounds: Potassium iodid was used in five per cent solution, one c.c. of this solution (.050 gm. KI) generally being given per 100 gms. of animal weight as the standard dose. Iodoform was used in 10 per cent emulsion in olive oil, in doses of about one c.c. per 100 gms. animal weight. This was found to be more toxic than the other iodin compounds, especially for pregnant animals in which abortions usually resulted. Iodipin, 25 per cent iodin, was given in doses of about 0.5 c.c. per 100 gms. Ethyl iodid (Merck) was given in doses of about one c.c. per kilo. After the designated time had elapsed, the animals were bled from the carotids as thoroughly as possible, and in removing the tissues for analysis care was taken not to have errors arise from contamination with fluid from the site of the injection, which was always located as far as possible from the tissues that were to be examined. The tissues were finely ground in the fusion mixture, dried, and kept until ready for analysis. The large excess of alkali present in the fusion mixture seems, in view of the excellent results obtained in the subsequent analyses, to have been adequate to prevent any loss of volatile iodin compounds, even of the highly volatile ethyl iodid.

At first we made our analyses by the method devised by Hunter,[1] which possesses very evident advantages over the Baumann method, in that exact titration is used instead of the colorimetric method with its large subjective element, and especially

[1] *Jour. Biol. Chem.*, 1910, 7, p. 321.

in that it involves estimation of six times the amount of iodin originally present, which enormously reduces the error in determining iodin in small fractions of a milligram, as is necessary in this work. We soon found, however, that while we had no trouble in obtaining accurate results in our trial analysis of thyroid tissue or of test mixtures, yet during actual series of analyses there frequently occurred serious errors, sometimes total loss and sometimes large excess. In a number of instances these errors destroyed a large amount of work because they involved essential members of a series where duplicates were impossible. Investigation showed that there are several possible sources of error in this method. These have also been noted by others, especially by F. C. Kendall of New York, who published a preliminary report of an improved method[1] and who very kindly furnished us with a detailed account of his observations before their publication. Not securing altogether satisfactory results even with this method, a systematic investigation of the sources of error and the best means of correcting them was made by one of us (Hedenburg) and a method was at last devised which has been found altogether reliable and which possesses the aforementioned advantages of Hunter's method. This method will soon be described in detail in another publication.

The main results of our experiments are given in Tables 1 and 2, in which the figures represent milligrams of iodin per gram of fresh weight of tissue or fluid. Where more than one figure is given, without explanation, the results of duplicate or triplicate analysis are concerned, and these indicate well the limits of accuracy of the methods used. A dash indicates that no analysis was made. A question mark indicates that there is doubt as to the accuracy of the result obtained.

DISCUSSION OF RESULTS.

Examination of Tables 1 and 2 discloses the following facts: The methods of analysis used are sufficiently delicate and accurate nearly always to give reliable results, even with the small fractions of milligrams of iodin present in many of the samples analyzed. Occasionally, a result was obtained which was obviously entirely incorrect, and this is indicated in the table by a question mark. Such errors were observed chiefly when the original Hunter method was used, rarely with the newly devised modification of Hedenburg.

The relative amount of iodin found in the various tissues and organs seemed to show some variation with the form in which the iodin was compounded when injected, whether water-soluble KI or lipoid-soluble CHI_3, iodipin or ethyl iodid. But the results are not altogether in harmony with the observations of Loeb and others on the organotropic character of the partition of different forms of iodin compounds. We do not attempt to interpret these disagreements, as our problem lies elsewhere, but give the figures without

[1] *Proc. Soc. Exp. Biol. and Med.*, 1910, 8, p. 120; complete report in *Jour. Amer. Chem. Soc.*, 1912, 34, p. 894.

further comment. In a few experiments, extraction was performed with absolute ether, and the tissues were prepared for extraction by grinding to powder with anhydrous sodium sulfate. The ether extract was then shaken out with water to remove any traces of iodides and evaporated to dryness after mixing with fusion mixture. It will be noted that a little iodin was found in the brain and fat tissues even when it was given in the form of KI. No differences were observed between the results with rabbits and with guinea-pigs.

Potassium iodid given subcutaneously is eliminated rapidly, so that there is very little left in the blood or tissues even 12 hours later. With iodoform and iodipin very irregular results were obtained, which perhaps depend upon local conditions modifying absorption. Thus, in an experiment with iodipin, none at all could be found in the blood or organs of one animal (19, Table 1), while other animals with somewhat larger doses showed considerable amounts. Also, of three guinea-pigs given iodoform in much the same way, the amount of iodin in the blood was respectively 0.205, 0.006, and 0.120 mg. per cubic centimeter of blood. We have not attempted to determine how large a proportion of the iodin found in the blood and tissues is in the form injected and how much is inorganic iodids.

The blood practically always contains more iodin, no matter in what compound it is given, than any tissue or organ, whether normal or otherwise. Occasionally during active excretion the kidney will be found to contain more iodin than the blood, and generally it contains nearly as large a proportion weight for weight, as the blood of the same animal. The relation is well shown in the following parallel columns from a series of analyses under various conditions of dosage, time, iodin compound, and kind of animal used (guinea-pigs and rabbits).

On account of its function of excreting iodin the kidney cannot be compared with other tissues. Of the chief viscera, the liver seems to be perhaps the most stable as to the proportion of iodin, but as a rule it contains much less iodin than the blood, generally about one-third as much per gram. Thus of 29 analyses under the varied conditions of these experiments, in three the liver contained approximately the same amount of iodin as the blood, in four it contained

TABLE 1.

IODIN IN NORMAL AND TUBERCULOUS TISSUES.

Animal	Dosage	Interval between Injection and Death	Blood	Liver	Spleen	Kidneys	Lungs	Tuberculous Glands	Miscellaneous	Autopsy Findings and Remarks
1. (Guinea-pig) 770 gms. .	0.385 gm. KI	6 hrs.	?	0.064 (left) 0.072 (right)	0.094	0.115	?	0.092	Testes, 0 110 Urine, 19.0 mg.	KI injected intravenously (in all other experiments subcutaneously). Lymph glands hard, large, full of small tubercles, some caseous; analyzed together, wt. 5.9 gms. Spleen riddled with tubercles, many smaller ones in liver, a few in lungs.
2. " 600 gms. .	0.300 gm. KI	48, 24, and 6 hrs.	0.179 0.218	?	0.074	0 167	0.151	0.160	Testes, 0 121	Glands large and caseous in inguinal region, hard and large in mediastinal, pelvic, and pancreatic; analyzed together, wt. 6 gms. Many 2–3 mm. tubercles in spleen, smaller ones in liver.
3. " 480 gms. .	0.250 gm. KI	48, 24, and 6 hrs.	0.337	0 108 (left) 0 110 (right)	0.071	0 034	0.147	0.203	....	Inguinal glands only slightly enlarged and caseous, pancreatic and mediastinal enlarged and hard; analyzed together, wt. 7.6 gms. Extensive miliary tubercles in liver and lungs, larger in spleen. Bled incompletely.
4. " 400 gms. .	0.200 gm. KI	72, 48, 24, and 6 hrs.	0.388 0.433	0.365 (left) 0.433 (right)	lost	0.518	0.348	0.295 0.481 pus	....	Inguinal glands contained softened material analyzed separately (0 9 gm.) from the rest of the enlarged glands (5 5 gms.). Many 1–2 mm. tubercles in spleen, a few in lungs and liver.
5. " 720 gms. .	0.350 gm. KI	48, 24, and 6 hrs.	0.680 0.430	0.567 0.552 0.635	0.108	1.168	0.213	0.285 wall 0.790 pus	....	Large caseous axillary glands containing 3.3 gms. softened material analyzed separately from the wall of the cavity, wt. 2.2 gms.
6. " 370 gms. .	0.2 gm. KI	6 and 3 hrs.	0.558	0.161	.....	.....	.....	0.369	....	Large caseous cervical glands.
7. (Rabbit) 1,750 gms. . . .	0.9 gm. KI	4 hrs.	0 479 0.472	0.132 (left) 0.133 (right)	.....	.....	0.290	.....	Eye, normal 0.220 tuberculous 0.381 Brain 0.020 Fat 0 032	Extensive tuberculosis of chorioid, this eye weighing 4.5 gms. and containing 1.7 mg. iodin; normal eye wt. 2.2 gms. iodin 0.48 mg. No tuberculosis elsewhere.
8. " 1,500 gms. . . .	0.750 gm. KI	6 hrs.	0.171 0.168	0 025 (left) 0.035 (right)	0	0.151	0.031	.....	Eye, normal 0.182 tuberculous 0 150	Moderate tuberculosis of chorioid, eye weighing 3.6 gms. and containing 0.541 mg. iodin; normal eye wt. 2 7 gms., iodin 0 492 mg. No tuberculosis elsewhere.
9. " 1,800 gms. . . .	0.9 gm. KI	8 hrs.	0.194 0.179	0.042 (left) 0.040 (right)	.....	.....	0.003	.....	Eye, normal 0.078 tuberculous 0.166 Brain 0 Fat 0.015	Tuberculous eye weighed 4.0 gms. iodin 0.67 mg.; normal eye wt. 3.2 gms., iodin 0.25 mg. No tubercles in other organs.
10. " 1,500 gms. . . .	0.750 gm. KI	12 hrs.	0.006 0.011	0	0	0	0	.....	No iodin in either eye	Normal eye wt. 2.8 gms., tuberculous, wt. 4.8 gms. Not sufficient iodin to be detected in any of the organs.

11. (Guinea-pig) 550 gms. .	5.5 c.c. 10 per cent CHI_3	48 hrs.	.....	0.128 (left) 0.071 (right)	0.100	0.111	0.100	0.152	....	Pregnant, died without being bled. Large caseous inguinal glands, 3.0 gms. Many miliary tubercles in spleen, a few in liver and lungs.
12. " 680 gms. .	6.8 c.c. 10 per cent CHI_3	72 and 24 hrs.	0.093 0.093	?	0.079	0.118	0.087	0 126	Muscle, 0.064	Bled fairly well. Large partly caseous inguinal and hard pancreatic and mediastinal glands analyzed together, wt. 6.1 gms. Liver very fatty. Spleen full of tubercles.
13. " 800 gms. .	4 c.c 10 per cent CHI_3	6, 5, 3 days	0.205	0.060 0.033 tubercles	0.013	?	0 090	0.013	Fat 0.052	Advanced pregnancy. Not well bled. Some large caseous areas isolated from rest of liver tissue and analyzed separately (0.8 gm.). Liver fatty. Spleen full of tubercles.
14. " 600 gms. .	3 c.c. 10 per cent CHI_3	5 and 3 days	0.006	0.002 0.002	0	0	0.003	0.007 capsule 0.013 pus ether extract 0	Testes 0 Fat 0	Inoculated in left testicle; both testicles found full of small tubercles. Few tubercles in liver. Large abscess in left axilla (pseudotuberculosis?) the pus of which was extracted with ether and extraction and residue analyzed separately.
15. (Rabbit) 1,600 gms. .	5 c.c. 10 per cent CHI_3	5 and 3 days	0.120 0.121	0.025 0.002 ether extract	.....	.....	.....	.....	Eye, normal 0.038 tuberculous 0.267	Inoculated in right eye, which was nearly ready to rupture, wt. 4.8 gms., iodin 1.28 mg.; normal eye wt. 4.0 gms., iodin 1.15 mg. No tuberculosis elsewhere.
16. " 2,000 gms. .	10 c.c. 10 per cent CHI_3	40 hrs.	0.217	0.172 (left) 0.180 (right)	.....	.....	0.126	.....	Eye, normal 0 093 tuberculous 0.117 Brain 0.069 Fat 0.279	Died from iodoform, and not bled, therefore all organs full of blood. Normal eye, wt. 3.0 gms., total iodin 0.279 mg.; tuberculous eye wt. 4.8 gms.; iodin 0.578 mg. No tuberculosis elsewhere.
17. (Guinea-pig) 550 gms. .	3 c.c. iodipin	72, 36, and 12 hrs.	0.019 0.031	0.080 (left) 0.053 (right)	0.061	0.195	0.032	0.111	....	Inguinal glands very large and caseous, some caseation in pancreatic and mediastinal glands; analyzed together, wt. 11.5 gms. Spleen almost entirely tuberculous, slight in liver and lungs. Thoroughly bled.
18. " 600 gms. .	3 c.c. iodipin	72 and 24 hrs.	0.082	0.093 (left) 0 091 (right)	0.052	0.016	0.086	0.044	....	Glands showed only slight caseation; wt. together 4.9 gms. Not much tuberculosis in other organs. Bled thoroughly.
19. " 600 gms. .	1 c.c. iodipin	4, 3 and 1 day	0	0	0	0	0	0	Testes 0	Inoculated in testicles. Extensive tuberculosis in all organs, especially lungs. No iodin could be found anywhere.
20. (Rabbit) 1,750 gms. .	4.5 and 2.25 c.c. iodipin	3 and 1 day	0	0.009 (left) 0.008 (right)	.....	.....	0.021	.....	Eye, normal 0 0075 tuberculous 0 0062 Brain 0 Fat 0.045	Tuberculous eye wt. 5.2 gms., iodin 0 033 mg.; normal eye 2 0 gms., iodin 0.015 mg. No other tuberculosis.
21. " 1,700 gms. .	2 5 c.c. ethyl iodid	6 hrs.	0.215 0.207	0.042 (left) 0 036 (right)	.....	.....	0.080	.....	Eye, normal 0 028 tuberculous 0.148 Brain 0.003 Fat 0	Normal eye wt. 2 2 gms., iodin 0.062 mg.; tuberculous eye 5 8 gms. iodin 0.86 mg. No tuberculosis elsewhere.

TABLE 2.

IODIN IN NECROTIC TISSUES AND EXUDATES.

Animal	Dosage	Interval between Injection and Death	Blood	Liver	Spleen	Kidneys	Lungs	Agar	Muscle	Exudate	Miscellaneous	Autopsy Findings and Remarks
1. (Guinea-pig) 370 gms.	0.38 gm. KI	6 hrs.	0.488	0.101	0.139	0.248	0.312	0.265	0.087	0.221	Bile 0.311	8 c.c. agar injected subcutaneously 60 hrs. before bleeding, which was incomplete. Agar found well encapsulated; much edema in surrounding tissue, which was analyzed separately.
2. " 450 gms.	0.15 gm. KI	6 hrs.	?	0.077 (left) 0.090 (right)	0.107	0.111	0.112	0.100	.....	.....	Bile 0.310 Glands 0.218	Duplicate of No. 1, but not bled until 6 days after agar injection.
3. " 720 gms.	0.35 gm. KI	18, 24, and 6 hrs.	0.680	0.635 0.567 0.552	0.108	1.168	0.213	0.280	....	....	Capsule about agar 0.390	Duplicate of Nos. 1 and 2, bled after 9 days.
4. (Rabbit) 3,450 gms.	1.5 gm. KI	18 and 1 hr.	0.812	0.485 (left) 0.513 (right)	0.365	0.670 (left) 0.420 (right)	0.420	0.435	0.130	..	Abscess pus 0.503	Left kidney ligated 3 days and agar injected 9 days before death, and also agar and staphylococci to produce subcutaneous abscess. Died without being bled. Advanced pregnancy. Liver very cirrhotic. Left kidney entirely necrotic, wt. 23.2 gms., contained 15.7 mg. iodin; right kidney wt. 11.4 gms.; iodin 4.8 mg. Sterile agar well encapsulated; infected agar in abscess cavity.
5. " 2,140 gms.	1.06 gm. KI	18, 24, and 6 hrs.	0.524 0.536	0.187 (left) 0.188 (right)	0.317	0.487 (left) 0.500 (right)	....	.....	0.095	..	Hematoma in muscle 0.352	Left kidney ligated 5 days before death. Bled only fairly thoroughly. Left kidney entirely necrotic, wt. 11.2 gms., iodin 5.4 mg.; right kidney 5.2 gms., iodin 2.6 mg. Hematoma in psoas muscle analyzed separately.

6. (Rabbit) 2,470 gms...	1.24 gm. KI	25 and 6 hrs.	0.425	0.114 (left) 0.131 (right)	0.178	0.539 (left) 0.389 (right)	0.040? (left) 0.147 (right)	.....	0.061	0.159	....	Left kidney ligated 5 days before bleeding; 3 days later injected aleuronat into left pleural cavity. Bled well. Much turbid exudate and fibrin in pleura, analyzed together, wt. 11 gms. Left lung collapsed and congested. Left kidney necrotic, wt. 14.2 gms.; iodin 6.7 mg.; right wt. 9 gms., iodin 3.5 mg.
7. " 1,500 gms...	0.75 gm. KI	24 and 6 hrs.	0.391	0.681 (left) 0.894 (right)	0.221	0.267 (left) 0.319 (right)	0.184	.....	.....	0.343	....	Duplicate of No. 6. Bled poorly. Resembled No. 6 at autopsy. Left kidney 9.7 gms., iodin 2.6 mg.; right, 6.6 gms., iodin 2.1 mg.
8. " 2,000 gms...	1.0 gm. KI	22 and 6 hrs.	0.303 0.306	0.095 (left) 0.105 (right)	0.123	0.403 (left) 0.530 (right)	0.326 (left) 0.189 (right)	.....	0.030 normal 0.300 necrotic	0.310 edema 0.361 pleura 0.461 fibrin in pleura	....	Kidney ligated 12 days; formalin injected into muscle 4 days and aleuronat - staphylococcus suspension into left pleura 2 days before bleeding thoroughly. Heavy fibrino-purulent pleuritis and pericarditis; separated fibrin from fluid and analyzed separately. Left lung collapsed. Left kidney necrotic, wt. 30.5 gms., iodin 12.3 mg.; right kidney 8.2 gms., iodin 4.35 mg. Muscle necrotic where formalin injected; much subcutaneous edema fluid, analyzed separately. Focal necrosis in liver.
9. (Guinea-pig) 620 gms	0.31 gm. KI	3 days, 29 hrs.	0.0	0.007	0.010	?	0.011	0.020	.....	.....	Fat 0.030 Brain 0.030	Agar injected 9 days before bleeding. Necrosis of skin followed first KI injection.
10. " 600 gms	0.3 gm. KI	4 and 2 days	0.005 0.006	0.004	0.079?	?	0.016	0.022	.....	.....	....	Duplicate of No. 9, but killed 1 day later.

TABLE 2—*Continued.*

Animal	Dosage	Interval between Injection and Death	Blood	Liver	Spleen	Kidneys	Lungs	Agar	Muscle	Exudate	Miscellaneous	Autopsy Findings and Remarks
11. (Rabbit) 1,400 gms...	0.35 gm. KI	36 and 12 hrs.	0.016 0.023	0.008 0.007	?	0.020 (left) 0.011 (right)	0 016	0.916?	0.004 normal 0.017 necrotic	0 020 pleura 0 014 edema	....	Kidney ligated 8 days, aleuronat injected subcutaneously and formalin injected into muscle 3 days, agar injected 6 days before bleeding thoroughly. Aleuronat found encapsulated. Muscle necrotic and much subcutaneous edema where formalin was injected; fluid analyzed separately. Left kidney wt. 11 9 gms., iodin 0 24 mg.; right 6 9 gms., iodin 0 07 mg.
12. " 1,800 gms...	5 c.c. 10 per cent CHI_3	3 days	.....	0.014	.....	0.021 (left) right lost	.....	0.012	0.001 normal 0.091 necrotic	.....	Brain, ether extract 0 Residue 0.025	Kidney ligated 6 days. Agar injected 5 days; emulsion of aleuronat and turpentine injected into pleura, and formalin into leg 3 days before death. Not bled. Brain extracted with ether.
13. " 1,700 gms...	5 c.c. 10 per cent CHI_3	6 days	0.072 0.072	0.032	?	0.099 (left) 0.069 (right)	0 088 (left) 0 071 (right)	0.077	0 013 normal 0.100 necrotic	0 078 pleura	Bile 0.014 Fat 0.076	Duplicate of 12, except lived 2 days longer and then was bled to death. Severe left pleurisy, with atelectasis of left lung. Liver very fatty. Muscle necrosed by formalin. Left kidney wt. 12.8 gms., iodin 1 26 mg.; right 6.9 gms., iodin 0 47 mg.
14. " 1,500 gms...	3 c.c. 25 per cent iodipin	6 days	0.006 0.007	0.002 0.002	0	0.001 (left) 0.002 (right)	0.001	0.010	0.0 normal 0.001 necrotic	0 0035 pleura 0 0025 edema	Brain 0 Fat 0.005	Ligated kidney, 8 days, agar injected 7 days, aleuronat and turpentine emulsion injected into chest-wall and formalin injected into leg 3 days before being bled to death. Much subcutaneous edema near formalin-killed muscle. Left kidney necrotic, wt. 11.4 gms., iodin 0.009 mg.; right 6.2 gms., iodin 0.012 mg.

15. (Rabbit)	1,750 gms. . .	1.75 c.c. ethyl iodid	6 hrs.	0.168 0.161	0.060 0.052	0.030	0.066 (left) 0.256 (right)	0.043	0.072	0.018 normal 0.052 necrotic	0.105 aleuronat 0.130 edema	Brain 0.001 residue Ether extract 0	Duplicate of No. 11. Aleuronat injected into peritoneum and removed as solid dry lump. Brain removed and ether extract analyzed. Left kidney wt. 11.1 gms., iodin 0.8 mg.; right 5.1 gms., iodin 1.3 mg.
16. "	2,000 gms. . .	2 c.c. ethyl iodid	6 hrs.	0.136 0.124	0.039 0.042 0.020 ether extract	. . . . .	0.026 (left) 0.120 (right)	0.019 (left) 0.020 (right)	0.024	0.012 normal	0.090 fluid 0.066 solid	Brain, ether extract 0	Duplicate of No. 15, except aleuronat and turpentine emulsion was injected into left pleura, causing much exudate and collapse of left lung and some solidification, fibrin of exudate analyzed separately from fluid. Liver showed extensive focal necrosis. Left kidney, wt. 14.8 gms., iodin 0.4 mg.; right 6.2 gms., iodin 0.75 mg.
17. "	2,500 gms. . .	2.5 c.c. ethyl iodid	12 hrs.	0.433	0.102 (left) 0.097 (right)	0.079	0.214 (right) left lost	0.320 (left) 0.230 (right)	0.283	0.040 normal 0.064 necrotic	0.336	Brain 0.021	Duplicate of No. 16 but died when bleeding was started, and therefore incompletely bled. No focal necrosis of liver.

a very little more and this when there was but very little iodin present (three were iodipin and one KI experiments); while of the 22 in which the blood contained the most iodin, in 16 the excess was in a ratio between two and four to one.

TABLE 3.

RELATIVE IODIN CONTENT OF KIDNEYS AND BLOOD.

	Blood	Kidney	Injection	Time Elapsed after Injection
1	.488	.248	KI	6 hours
2	.680	1.168	KI	48, 24, and 6 hours
3	.812	.420	KI	18 and 1 hour
4	.524	.500	KI	48, 24, and 6 hours
5	.425	.389	KI	24 and 6 hours
6	.391	.319	KI	24 and 6 hours
7	.306	.530	KI	22 and 6 hours
8	.005	.000	KI	4 and 2 days
9	.019	.011	KI	36 and 12 hours
10	.093	.118	CHI_3	72 and 24 hours
11	072	.069	CHI_3	6 days
12	.025	.195	iodipin	72, 36, and 12 hours
13	.060	.016	iodipin	72 and 24 hours
14	.130	.120	C_2H_5I	6 hours
15	.432	.214	C_2H_5I	12 hours
16	.168	.256	C_2H_5I	6 hours

The statement of Loeb that the left lobe of the liver regularly contains less iodin than the right we can corroborate in part only. In only two of 19 livers examined was there appreciably more iodin in the left, and in 10 there was definitely more in the right. In the remainder there was no difference above the limit of error of analysis. Generally, the ratio varies between 5:6 and 7:8. The two exceptional results were obtained with iodoform and iodipin.

As a rule, we found less iodin in the lungs than in the liver, but often the amount is about the same, and it is not uncommon to find more in the lungs, especially when only traces are left in the body. In four experiments when ethyl iodid had been given, we observed an excess of iodin in the lungs in two, and in the liver in two, which does not entirely corroborate Loeb, but our figures are too few to be significant.

The figures for the spleen vary greatly, perhaps because of the small quantity of material available for analysis; all in all it ranked about the same as in the liver. Herein we fail to corroborate Boruttau, who states that lymphatic tissues take on an excess of iodin, but corroborate Loeb (1912).

In all cases the muscle content runs far below that of all the

other tissues, except the brain, containing usually but one-half to one-third as much as the liver. Thus, the average of 12 analyses of liver and muscle from the same animals under varying conditions, showed 0.115 mg. iodin per gram liver and 0.041 mg. per gram muscle. In only two cases did the muscle have as much as half the amount present in the liver. The testicles seem to take on about as much iodin as the liver, and apparently the bile is an important avenue of escape of iodin from the blood.

The effects of pathological changes upon the tissues were very definite. Tuberculous lymph glands do, as Loeb first showed, take up in general relatively more iodin from the blood than do the liver, spleen, and lungs of the same animal. Thus, in nine of 11 experiments the tuberculous lymph glands contained more iodin than the liver, and in the best experiments with KI the amount approaches that in the blood. See Table 4.

TABLE 4.
IODIN IN CASEOUS GLANDS.

No. in Table 1	Blood	Liver	Caseous Glands	Injection	Time after Injection
1	?	.068	.092	KI	6 hours
2	.195	?	.160	KI	48, 24, and 6 hours
3	.337	.109	.203	KI	48, 24, and 6 hours
4	.408	.400	.481	KI	78, 42, 24, 6 hours
5	.550	.580	.790	KI	48, 24, and 6 hours
11	...	.100	.152	CHI_3	48 hours
12	.093	....	.126	CHI_3	72 and 24 hours
13	.205	.060	.013	CHI_3	6, 5, and 3 days
14	.006	.002	.013	CHI_3	5 and 3 days
17	.025	.065	.111	iodipin	72, 36, and 12 hours
18	.082	.092	.044	iodipin	72 and 24 hours
19	.0	.0	.0	iodipin	4, 3, and 1 day

It is especially noticeable that when the caseous material was abundant enough to permit of separation from the rest of the gland substance, it contained much more iodin than did the non-caseous portion of the glands, as seen in experiments Nos. 4, 5, and 14, where the figures are:

	4	5	14
Gland substance	0.295	0.285	0.007
Caseous contents	0.481	0.790	0.013

In only a few instances was there a noticeable deficit in iodin in the tuberculous tissues. In experiment No. 13 a small amount of necrotic liver tissue (0.9 gm.) seemed to contain less iodin than the

rest of the liver, but the amount of iodin involved is so small that the results are of doubtful reliability. The fact that here and in the glands in Nos. 13 and 18 the amount of iodin is lower in the caseous tissue than in the normal liver, may be ascribable to a relative chemotropism of the liver for iodoform and iodipin used in these experiments.

Tuberculous lesions in the eye show, as was also found by Loeb and Michaud in four experiments, an increased capacity for taking up iodin, as shown by the following summary from Table 1.

TABLE 5.

IODIN IN TUBERCULOUS AND NORMAL EYES.

No. in Table 1	Weight of Eye		Total Iodin		Mg. Iodin per gram		Form of Iodin Injected
	Normal	Tuberculous	Normal	Tuberculous	Normal	Tuberculous	
7	2.2	4.5	0.48	1.7	0.220	0.381	KI, 4 hours
8	2.7	3.6	0.49	0.54	0.182	0.150	KI, 6 hours
9	3.2	4.0	0.25	0.67	0.078	0.166	KI, 8 hours
10	2.8	4.8	0	0	0	0	KI, 12 hours
15	4.0	4.8	0.15	1.38	0.038	0.267	Iodoform
16	3.0	4.8	0.28	0.58	0.093	0.117	Iodoform
20	2.0	5.2	0.015	0.033	0.0075	0.006	Iodipin
21	2.2	5.8	0.062	0.86	0.028	0.148	Ethyl iodid
Average	2.76	4.7	0.216	0.720	0.081	0.154	

Of these eight experiments, without exception the amount of iodin is greater in the tuberculous eye than in the normal eye, although in two (8 and 20) the proportion of iodin is slightly greater in the normal eye. Taken all together, there is over three times as much iodin in the tuberculous eyes, and nearly twice as large a proportion. The low figure for iodipin (No. 20) corresponds entirely with the proportion of iodipin in the liver of the same animal (0.008), and it is evident that after injections of iodoform and ethyl iodid the iodin readily enters the eyes, especially the tuberculous eyes, although whether as organic or inorganic compounds we have not ascertained.

That the entrance of iodin into tuberculous tissue is not characteristic of tuberculosis is established by the analyses of the tissues of animals in which necrosis and exudates were produced experimentally (Table 2). In 10 rabbits which had the left kidney rendered totally necrotic by ligation of all the blood vessels, there

is found to result a great increase in the size of the organ, from an average of 7.2 gms. to 15.3 gms., because of hemorrhage and edema. In spite of the avascularity of these kidneys, iodin permeates them rapidly, so that six hours after injection there is found to be, on the average, almost identically the same proportion of iodin in the avascular necrotic kidney and in the normal kidney, a proportion which, as pointed out previously, approximates that of the iodin content of the blood more closely than in any other organ. These facts are shown in Table 6, summarized from Table 2. Therefore, it seems evident that in a short time, the iodin in the blood will penetrate even so large an avascular area as an entire kidney, and reach practically the same concentration as in the blood itself.

TABLE 6.

Iodin in Normal and Necrotic Kidneys.

No. in Table 2	Gms. Weight of Kidneys		Total mg. Iodin in Kidneys		Mg. Iodin per gm. of		
	Necrotic	Normal	Necrotic	Normal	Blood	Necrotic Kidney	Normal Kidney
4	23.2	11.4	15.7	4.8	.812	.679	.420
5	11.2	5.2	5.4	2.6	.530	.487	.500
6	14.2	9.0	6.7	3.5	.425	.539	.389
7	9.7	6.6	2.6	2.1	.391	.267	.319
8	30.5	8.2	12.3	4.4	.305	.403	.530
11	11.9	6.9	0.2	0.1	.019	.020	.011
13	12.8	6.9	12.6	4.7	.072	.099	.069
15	12.1	5.1	10.8	1.3	.168	.066	.256
16	14.8	6.2	0.4	0.8	.130	.026	.120
Total	140.4	65.5	66.7	24.3	2.852	2.586	2.614
Average	15.6	7.3	7.4	2.7	.317	.287	.290

Of all the tissues, however, the normal kidney alone seems to be so permeable for iodin that it comes to contain the same proportion as the blood, a fact which is presumably related to the functional activity of the organ. If we take another tissue which is not normally so permeable for iodin, such as the muscle, we find the interesting fact that necrotic areas in this tissue also tend to contain approximately as much iodin as the blood of the same animal, while the normal muscle tissue, in spite of its much greater blood supply, contains much less iodin. This fact is shown in Table 7.

In the above series the necrosis was produced by injection of strong formalin (except No. 5, in which trauma during operation

probably caused the injury). In removing the tissues at autopsy, the necrosis not being sharply circumscribed, more or less normal tissue was probably always included with the necrotic muscle; if only completely necrotic muscle had been present in these samples, it is probable that the proportion of iodin would have been still higher.

TABLE 7.

IODIN IN NORMAL AND NECROTIC MUSCLE.

No. in Table 2	Blood	Normal Muscle	Necrotic Muscle
1	.488	.087	....
4	.812	.139	....
5	.530	.095	.352
6	.425	.061	..
8	.305	.030	.300
11	.019	.004	.017
12	..	.001	.091
13	.072	.013	.100
14	.006	.000	.001
15	.165	.018	.052
16	.136	.012	...
17	.433	.040	.064
Average of analyses where all three figures were obtained	.219	.029	.126

The explanation of these results, it seems to us, must be as follows: The partial impermeability of living cells, which presumably differs in all organs and cells, is destroyed when the cell is killed. Therefore, the readily diffusible iodin compounds present in the blood and tissue fluids will diffuse into the necrosed tissue elements just as they would into any inert water-filled colloidal mass, with the resulting tendency, as shown by our figures, to approach osmotic equilibrium of iodin in blood and necrotic tissue. The large amount of iodin present in necrotic tissues, whether tuberculous or otherwise, is, therefore, dependent on purely physical conditions, i.e., the destruction of the semi-permeability of the cells. That it does not depend upon any chemical attraction, or even a specific physical "adsorption," is shown by the fact that if some time is allowed for the iodin to be excreted in part from the body after injection, it leaves the necrotic tissues, the blood and the normal tissues *pari passu*. Support is given to this interpretation by the results of implantation of agar into the subcutaneous tissue, followed by injection of iodin compounds at various intervals. The agar was introduced at a temperature of about 50° C.,

and solidified in a lump which soon became encapsulated, and after a time permeated by invading strands of granulation tissue. The results of analyses from several such experiments are given in Table 8.

TABLE 8.

IODIN IN AGAR.

No. in Table 2	Blood	Liver	Agar
1	.488	.101	.265
2	....	.089	.100
3	.680	.580	.280
4	.812	.500	.435
9	....	.007	.020
10	.005	.004	.022
12	...	.014	.012
13	.072	.032	.077
14	.006	.002	.010
15	.168	.056	.072
16	.013	.040	.024
17	.433	.100	.283

These experiments seem to show a marked permeability of agar for iodin. In experiment No. 4, for example, although the tissues were examined only one hour after injection of the iodid, yet even this quickly the avascular agar contained as much iodin as the liver, which in this case gives an abnormally high figure because the animal could not be bled. In eight of 12 experiments, the agar contains a larger proportion of iodin than the liver, in only one is it considerably less. It will be noticed, also, that in this series as in all the other experiments the form in which the iodin is introduced seems to make little difference in its distribution.

As is to be expected, inflammatory exudates are prone to approach the blood in iodin content. Table 9 shows no evidence of any selective tendency of iodin to enter the inflammatory exudate, however, there nearly always being somewhat less iodin in the exudate than in the blood. The presence of iodin in the exudate would seem in all cases to be dependent entirely on simple diffusion, as in the case of necrotic tissues and implanted agar.

The high iodin content of tuberculous eyes is presumably to be explained, therefore, as due in part to the inflammatory exudate present in these eyes, and probably in less degree, to the necrosis in the tuberculous tissues.

Similarly, compression atelectasis of the lung, produced by pleural exudates, with the resulting greater or less edema and

inflammatory exudate in the alveoli, is associated with a corresponding slight increase of iodin in the injured lung, as shown in Table 10.

TABLE 9.

IODIN IN EXUDATES.

No. in Table 2	Blood	Liver	Exudate	Character of Exudate
1	.488	.101	.221	Edema about agar
4	.812	.500	.503	Subcutaneous abscess
6	425	.125	.159	Serofibrinous, pleural
7	.391	.720?	.343	" "
8	.305	.100	.310	Edema, subcutaneous
			.361	Pleural fluid
			.461	" fibrin
11	.019	.007	.020	Aleuronat subcutaneously
			.014	Edema, subcutaneous
13	.072	.032	.078	Serofibrinous pleuritis
14	.006	.002	.0035	" "
			.0025	Edema, subcutaneous
15	.165	.059	.130	" "
			.105	Aleuronat, intraperitoneal
16	.130	.040	.090	Pleural fluid
			.067	" fibrin
17	.433	.100	.336	Serofibrinous pleuritis

TABLE 10.

IODIN IN NORMAL AND COLLAPSED LUNGS.

No. in Table 2	Blood	Normal Lung	Collapsed Lung
6	.425	.147	.040?
8	.305	.189	.326
13	.072	.071	.088
16	.013	.020	.019
17	.433	.230	.320

These observations would seem to explain entirely, and on a simple physical basis, the observation of Bondi and Jacoby, Fillipi and Nesti, and Loeb, that drugs tend to enter inflammatory exudates. They enter simply because there is present an exudate which offers no resistance to their permeation to establish an osmotic equilibrium with the blood, and not because there is a specific affinity between pathological tissues and blood. Therefore, we are led to the conclusion that *the supposed affinity of certain drugs for certain pathological tissues merely depends on a decreased impermeability of the diseased cells, or diffusion into inflammatory exudates present in the diseased area, or both.* If this is the case, we might expect a non-diffusible colloidal substance to be unable to penetrate avascular diseased areas which are highly permeable for crystalloids, and this was found to be true. Tubercles, where there is no

blood supply, are relatively or absolutely impermeable, to foreign proteins present in the blood. This was shown in the following series of experiments.

ENTRANCE OF EGG ALBUMEN INTO TUBERCLES.

In order to determine the entrance of proteins into tubercles, advantage was taken of the delicate and accurate method offered by the anaphylaxis reaction for the detection of small quantities of foreign proteins. The following experiments were performed:

Experiment 1.—A large guinea-pig, which had been inoculated subcutaneously with 0.01 mg. of human tubercle bacilli three months previously, was bled to the amount of 4 c.c., and an equal amount of fresh filtered four per cent solution of Merck's dried egg albumen powder was injected into the blood by the intracardiac route. After three hours the animal was bled to death and the blood defibrinated. Autopsy showed large caseous cervical glands and smaller tuberculous mediastinal and inguinal glands. The spleen was studded with tubercles, many of which were two to four millimeters in diameter. The liver and lungs showed a few tubercles one to two millimeters in diameter. Samples were taken aseptically from various tissues, ground up with quartz sand, emulsionized under aseptic precautions in 10 c.c. of water for each gram of substance. After filtering, the extract was injected intraperitoneally in various sized doses into 22 guinea-pigs. Most of these pigs died in four to six days with a peculiar gelatinous exudative peritonitis, great numbers of cocci being present in this exudate. Eighteen days after injecting the tissue extract, the survivors received by the peritoneal route an injection of 0.05 gm. Merck's egg albumen, to discover whether the tissue extracts had contained sufficient egg albumen to sensitize the injected pigs. The results were as follows:

	Sensitizing Dose	Result of Second Injection
1.	5 c.c. blood (undiluted)	Died in 18 minutes
2.	1 c.c. " "	" " 15 "
3.	0.1 c.c. " "	" " 20 "
4.	0.5 c.c. urine (undiluted)	" " 45 "
5.	4 c.c. spleen extract	Severe symptoms, temperature fell to 100°
6.	1 c.c. " "	" " " " 96°
7.	1 c.c. extract of caseous glands	Slight " " " from 104° to 101°
8.	0.75 c.c. " " tubercles from liver	No definite symptoms. No fall in temperature
9.	0.50 c.c. " " " " "	No definite symptoms. Temperature 101°

On account of the large proportion of deaths from infection, the results of this series were not altogether satisfactory, but such experiments as could be completed indicated that there was less

sensitization by extracts of tuberculous liver tissue and caseous glands than by extracts of spleen tissue taken from between the tubercles.

Experiment 2.—In the second experiment the danger of peritonitis was avoided by making the sensitizing injections subcutaneously. A 400 gm. guinea-pig which had large caseous inguinal glands from injection of human tubercle bacilli, was given an injection of three cubic centimeters of a four per cent solution of egg albumen powder in the carotid artery. This animal was very sick when injected, and it was so nearly dead three hours later when bled to death that it could be but partly bled, only five to six cubic centimeters escaping; therefore, all the organs were left containing much blood, including the caseous lymph glands, the necrotic content of which, when removed by scraping, was somewhat blood tinged. The tissues and blood were extracted with 10 parts of water as before, and the extract was injected in doses of from one to five cubic centimeters into 19 guinea-pigs. In this experiment, the sensitizing dose chosen was evidently too large, since 18 of the animals reacted fatally to egg albumen 18 days later. It can only be stated that the animals sensitized with extracts of the caseous material did not die as quickly as the others, and the sole survivor was in this group. Presumably, the amount of blood present in the tissues was sufficient to produce a fatal sensitization in the doses used.

Experiment 3.—The difficulties disclosed in the two previous experiments were avoided in a third trial. Here a 400 gm. pig with a large mass of fluctuating tuberculous lymph glands received two cubic centimeters of a four per cent solution of Merck's egg albumen in the jugular vein. Three hours later it was bled to death, but bled poorly and much blood was left in the body. The liver was found riddled with small tubercles, the spleen was greatly enlarged and contained some good-sized necrotic areas. The inguinal glands contained a great amount of soft caseous material, part of which was removed in two separate portions without pressure and without appreciable contamination with blood. Specimens of blood, liver tissue, spleen tissue between the large tubercles, the two separate lots of caseous material, and the uncaseated peripheral gland substance itself were each ground with quartz sand, extracted six hours with repeated stirring in 10 volumes of sterile water, filtered, and the filtrate injected subcutaneously into guinea-pigs. After 18 days, each pig was injected intraperitoneally with .050 gm. Merck's egg albumen, with the results shown in the table on p. 371.

From these experiments, it seems evident that the egg albumen present in the circulating blood does not enter the caseous material which is shut off from the blood by proliferating tissue, during three hours after its intravascular injection, at which time the blood contains sufficient egg albumen to sensitize a guinea-pig when injected in a dose of 0.001 c.c.[1] It is not possible to tell whether

[1] It may be mentioned that our experiments differ radically in one result from those of Vaughan, Cumming, and McGlumphy (*Ztschr. f. Immunitätsf.* 1911, 9, p. 16), for they state that egg-white injected into the blood of rabbits disappears in one hour, although it may be found in the various organs after that time. We found that egg albumen injected into the blood of guinea-pigs remains in the blood at least three hours, when 0.001 c.c. of blood contains a sensitizing dose. How much longer than three hours the albumen remains in the blood, and how much smaller doses than 0.001 c.c. are capable of sensitizing, we did not determine.

the positive results obtained with the liver, spleen, and tuberculous glands depend on the egg albumen contained within the cells or that present in the blood in these tissues. We were unable to secure a sufficient number of guinea-pigs to investigate this point.

1. Blood		1 c.c.	Died in 20 minutes
2. "		0.1 c.c.	Severe reaction
3. "		0.02 c.c.	" "
4. "		0.01 c.c.	" "
5. Liver		2.0 c.c.	Moderately severe reaction
6. "		0.5 c.c.	Died after 2 hours
7. "		0.05 c.c.	" " " "
8. Spleen		1 c.c.	Moderate reaction
9. "		0.1 c.c.	Slight "
10. "		0.01 c.c.	" "
11. Gland tissue		1 c.c.	Died in 30 minutes
12. " "		0.1 c.c.	Moderate reaction
13. " "		0.01 c.c.	" "
14. Caseous material	Sample A.	2.0 c.c.	No reaction
15. " "	" "	0.5 c.c.	Slight or doubtful reaction
16. " "	" "	0.1 c.c.	Doubtful reaction
17. " "	" "	0.01 c.c.	No reaction
18. " "	Sample B.	2.0 c.c.	Doubtful reaction
19. " "	" "	0.5 c.c.	No reaction
20. " "	" "	0.1 c.c.	" "
21. " "	" "	0.01 c.c.	" "

SUMMARY.

A systematic consideration of the chemotherapy of tuberculosis rests on an investigation of the permeability of both the tubercle bacillus and the tuberculous lesion for chemical substances of different characters. It is shown that compounds of iodin injected into tuberculous animals enter glandular tubercles with readiness, so that the proportion of iodin in such tubercles is usually greater than it is in most other tissues except the kidney; furthermore it is greater in the caseous contents than in the cellular peripheries of the tubercles. Tuberculous eyes usually contain much more iodin than their normal mates. This property is shown not to depend on any specific character of the tubercle itself, for other

necrotic tissues also take up more iodin than normal tissues. The explanation offered is that normal cells are not perfectly permeable to iodids (except perhaps kidney cells) and lose this impermeability or semi-permeability when killed or injured, thus becoming entirely permeable for crystalloids present in the surrounding fluids. As the iodin content of the blood increases and decreases with absorption and elimination, so the iodin in the necrotic area, whether tuberculous or otherwise, varies, indicating an absence of any chemical or physical binding of the iodin in such areas. A simple, inert, colloid agar, implanted in the tissues, behaves in quite the same way.

Egg albumen injected into tuberculous pigs is found, by means of the anaphylaxis reaction, to penetrate the avascular tubercles but little if at all, even when present in the blood in large amounts. This agrees with the hypothesis that the passage of iodin from the blood into the tubercles is a purely physical matter, the crystalloidal iodin compounds diffusing through the inert colloidal solution of a necrotic area practically unimpeded, while the colloidal egg albumen, according to the law of colloidal diffusion, is practically unable to diffuse through such a colloidal solution.

No evidence could be found of any tendency for iodin compounds of whatever nature to accumulate in tubercles or other necrotic areas, or to persist in such areas when disappearing from the normal tissues and the blood.

Exudates contain approximately the same proportion of iodin as the blood of the same animals, and hence any area with inflammatory edema and congestion will commonly show more iodin than normal tissues, although not usually more than the blood. No evidence was found of any specific entrance or fixation of iodin in inflammatory exudates. The iodin is distributed about alike in the fluid and solid portions of the exudate, indicating simple diffusion. Of normal tissues only the kidney seems to contain approximately as much iodin as the blood of the same animal. This may have some bearing upon its excretory function, since it indicates a greater permeability of renal cells than of other gland cells for iodids.

INTRA-VITAM STAINING OF TUBERCULOUS GUINEA-PIGS WITH FAT-SOLUBLE DYES.*

STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS. II.

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HISTORICAL.

Several experimenters have investigated the power of fat stains, especially sudan III, to stain tissues within the living animal. A review of much of this work is furnished by Riddle,[1] who developed the important fact that this dye, when fed to laying hens, is deposited in the yolk within a short time. He also found that sudan III is almost, but not altogether, without harmful effects, there being observed certain defects in the feathers and a lowered resistance to starvation, which he was inclined to ascribe to a relative lack of availability of the stained fat for the metabolism of the animal. However, this degree of toxicity must be low, since it was found by the Gages[2] that eggs dyed in this way will hatch out normal chicks with stained body fats. Birds seem to be especially favorable subjects for this sort of work, depositing the dye abundantly and rapidly, though it can be taken up also by the fat tissues of mammals and reptiles. It seems that the dye accompanies the fat in which it is given or with which it is absorbed, so that it is deposited in fat depots and similar places that are lying on fat, but not in fatty tissues which are not lying on fat, e.g., the fat tissues of wasting animals, or in lipoid-rich tissues such as the central nervous system. Neither does the dye seem to enter the normal fatty lipoid constituents of the parenchymatous cells of the viscera. When the fat dye is given to guinea-pigs by itself, not dissolved in fat, it does not color even the depot fats appreciably; likewise scarlet R., when given in vaseline or lanolin, substances unavailable for metabolism, does not become deposited in the tissues (Jacobsthal[3]). According to Mendel and Daniels[4] the dye is excreted in the bile, in which it is very soluble, which probably explains the absence of the dye in livers with fatty degeneration experimentally produced by phlorhizin or phosphorus in animals whose fat has been stained with fat dyes (Jacobsthal, Mendel, and Daniels). Jacobsthal states that a certain amount of staining may occur in fat in the liver cells if the degeneration is produced by hunger and exposure, and very large doses of stained fat are given; and Mendel and Daniels found that when very large amounts of deeply stained fat are given there may be some staining in the liver. Schmorl[5] also found some staining of fat in necrotic renal cells.

From the above papers it seems evident that there is no marked tendency for sudan III deposited in the depot fats, or present in the food fats, to enter the fat droplets present in degenerated parenchyma cells, at least in the liver and kidney; but this

* Received for publication August 24, 1912.

[1] *Jour. Exper. Zoöl.*, 1910, 8, p. 164.

[2] *Anat. Record*, 1909, 3, p. 203.

[3] *Verhandl. deutsch. path. Gesellsch.*, 1909, 13, p. 380.

[4] *Proc. Soc. Exper. Biol.*, 1911, 8, p. 126.

[5] In discussion following Jacobsthal's paper.

negative result may be explained by the fact that in these two tissues we have the places of excretion of the dye, which may interfere with its storage, especially in the liver where we have an excess of bile in which the fat dyes are very soluble.

Other fat dyes seem to have received relatively little consideration as *intra-vitam* stains. Jacobsthal worked chiefly with scarlet R., which seems to behave much the same as sudan III. He failed to get results by intravenous injection of Nile blue sulfate, or by feeding indophenol.

Alkanna, which is a vegetable dye and not a synthetic product like most of the fat dyes, seems to behave much like sudan III when used as an *intra-vitam* stain, according to the results obtained by Hofbauer.[1] Mendel and Daniels[2] have recently described experiments with Biebrich scarlet, which gives results comparable to those obtained with sudan III; with indophenol-blue which gave negative results, attributed to the reduction of this dye in the body; with "oil-soluble green" and annatto which gave entirely negative results.

There seem to be but few recorded instances of the feeding of fat dyes to man, one being a case of chyluria studied by Franz and Stejskal.[3] They administered five grams of olive oil "mit Sudan III intensiv roth gefärbt," and found that the dye appeared in the urine. Hofbauer[4] gave sudan III in capsules to a woman twenty hours previous to a Cesarean section, and claims that he found the dye in both maternal and fetal blood. Davidsohn[5] records an interesting case of a child which died after being given a "carrot cure"; the body fat was found everywhere deeply stained a carrot color, and as the pigment of carrots, carotin, is known to be a fat-soluble dye this may be fairly considered an instance of specific *intra-vitam* fat staining.

As tubercles contain a certain amount of fat, associated with disintegrative changes in the cells, and especially because of the fatty character of the tubercle bacillus itself, a set of experiments was performed to ascertain to what extent fat dyes administered to living tuberculous animals might penetrate the tubercles and stain the fat therein contained; also, incidentally, to note what effect, if any, the fat dyes might have upon the progress and development of the tuberculosis. All the dyes used in these experiments were from Grübler.

EXPERIMENTAL.

Feeding Experiments with Sudan III.

A series of guinea-pigs were fed a one per cent solution of sudan III in peanut oil (10 c.c. per kilo body weight) every second day during the period of the experiment.

Experiment 1.—Sudan III was fed for 88 days, at the end of which time the animal died of tuberculosis, having been inoculated on the 47th day after feeding was begun.

[1] *Pflügers Archiv*, 1900, 81, p. 263.

[2] *Jour. Biol. Chem.*, 1912, 12, p. 71.

[3] *Ztschr. f. Heilk.* (inn. med.), 1902, 23, p. 441.

[4] Quoted by Mendel and Daniels.

[5] In discussion following Jacobsthal's paper.

The postmortem findings showed all the fatty tissues, the subcutaneous, mesenteric, omental, testicular, etc., stained pink. The local glands at the inguinal region where the primary inoculation of the tubercle bacilli was made, were markedly enlarged but stood out distinctly unstained in comparison to the surrounding pink fat. The spleen contained numerous foci of necrosis, a few such areas were found in the lungs and the liver, but none of these appeared pink. The salivary glands were unstained. The kidneys and adrenals were unstained, but the surrounding fat was pink. The brain and cord was also unstained. The pus obtained from the inguinal glands contained numerous tubercle bacilli stainable by carbol fuchsin, but sudan III had not stained them *in vivo*. Frozen sections of the liver, kidneys, and spleen did not reveal any sudan staining.

The different tissues from this animal were dried and mixed with anhydrous Na_2SO_4 and were then extracted in a Greene extraction apparatus[1] by means of ether for five to eight hours, with the following results (++ designates an intense red color, + a red color still perceptible and unquestionable, —no red perceptible):

Lung, ++ (aspiration of dye accounts for the large amount of dye present in the lung extract in this and other experiments)	Fat at head of testes, ++
Other body fats, ++	Liver, +?
Kidney, —	Testes, —
Heart (ventricle), —	

Experiment 2.—This guinea-pig received sudan III for 241 days, at the end of which time it was killed, having been inoculated with tuberculosis on the 130th day after feeding with sudan III was begun.

The postmortem findings are practically identical with those in Experiment 1, with only slight differences in the tubercular involvement.

Experiment 3.—This guinea-pig received sudan III every other day for 216 days, and died at the end of this time of tuberculosis, having been inoculated on the 130th day after feeding was begun.

As in the first two pigs the postmortem revealed sudan III staining in all the fatty tissues, and none perceptible in the parenchymatous organs or tuberculous tissues.

Experiment 4.—This guinea-pig received sudan III every other day for 123 days, and died at the end of this time of tuberculosis, having been inoculated on the 47th day after feeding was begun.

Except for slight differences in the distribution of the tuberculosis this animal did not differ markedly from the previous pigs in the distribution of the sudan III.

Experiment 5.—A guinea-pig received sudan III for 83 days, at the end of which time it was killed, having been inoculated with tuberculosis on the 47th day after feeding with sudan III was begun.

Postmortem examination revealed the stain in all the fatty tissues as had been found in the previous pigs. The parenchymatous organs were not stained. The liver and spleen contained numerous small foci of necrosis unstained by sudan III. The inguinal glands contained a large amount of caseous material without a trace of pink color.

Experiment 6.—A control guinea-pig not inoculated with tuberculosis, received sudan III for 241 days, at the end of which time the animal was killed.

[1] *Jour. Biol. Chem.*, 1910, 7, p. 503.

Postmortem revealed a well-nourished animal in contrast to the previous five pigs, which were all emaciated and poor in body fat. All the fats of the body, subcutaneous, mesenteric, testicular, at the head of the kidney, etc., were stained pink, differing from the previous pigs only in amount of fat present. Parenchymatous organs revealed no pathological changes and were not stained. The brain and cord were negative for stain.

Experiment 7.—A guinea-pig with advanced tuberculosis was fed sudan III in oil four times in five days, at the end of which time the animal died of tuberculosis.

Autopsy revealed no pink coloration of the fats of the body and the ether extract of the liver, lung, kidneys, and adrenals, caseous glands and spleen did not contain any sudan III. (The extractions were carried out as previously described for Experiment 1.)

Experiment 8.—A second guinea-pig with advanced tuberculosis was fed sudan III in oil seven times (2 c.c. of one per cent solution) in eight days, at the end of which time the animal died of tuberculosis.

Postmortem revealed a large caseous set of left inguinal glands which were not stained, and numerous necrotic areas in the spleen, liver, and lungs. The body fats were sparse (atrophy of fat) but where small amounts were found, as in the mesentery and at the head of the testes, these were a faint brown-pink tint. The lungs contained a large amount of sudan III (aspiration of stained fat during feeding). The necrotic areas in the liver and spleen were not stained. Adrenals, kidneys, gall bladder, etc., were not stained.

Results of extraction of the various tissues by ether (as previously described for Experiment 1) were:

Lungs, ++	Liver, ++? The color of the extract was a deep brown-red and appeared to be deeply stained by sudan
Testes and fat, +?	Gall bladder and contained bile, −
Spleen, −	Kidneys, −
Adrenals, −	Caseous glands, −

Experiment 9.—A third tuberculous guinea-pig was fed sudan III in oil eight times in nine days, at the end of which time the animal died of tuberculosis.

Autopsy revealed enlarged caseous glands in the left inguinal region, and caseous and necrotic material in the upper left thigh, neither of these containing any pink stain. The fatty tissues were not pink but rather brown in appearance (atrophy). At the head of the testicles the fat appeared of brownish-pink hue. The spleen was much enlarged and composed of numerous necrotic areas. The liver was nearly all necrotic, but unstained. Lung contained numerous necrotic areas but no visible stain. Gall bladder was distended but unstained. Adrenals, kidneys, and brain were unstained. Results of ether extraction were as follows:

Lungs, +	Gall bladder and contained bile, +? a deep brown-red color
Liver, −	Kidneys, −
Brain, −	Spleen, −
Adrenals, −	Testes and fat at head of testes, −?

Experiment 10.—A fourth tuberculous guinea-pig was fed sudan III in peanut oil 22 times in 26 days, at the end of which time the animal died of tuberculosis.

At autopsy were found numerous enlarged caseous lymph glands (local and general) unstained, the liver was one mass of necrotic areas (unstained), there were large necrotic areas in the spleen which contained no dye, and the lungs also contained numerous small necrotic areas. The fats of the body were markedly atrophied, so that practically no fat was found in the ordinary fat depots and no visible stain was found.

The results of ether extraction of the tissues of this animal were as follows:

Lungs, ++	Caseous lymph glands, –
Liver, –	Kidneys, –
Spleen, –	Adrenals, –

As a result of the above feeding experiments with sudan III in oil, the following conclusions seem justified:

1. Sudan III in oil, fed to normal guinea-pigs, stains all the body fats pink but does not visibly stain the parenchymatous organs of the body.

2. Sudan III dissolved in oil, fed to normal guinea-pigs which were later inoculated with tuberculosis and the feeding continued, also stained the body fats pink, but did not stain the tuberculous lesions (necrotic areas in the liver, spleen, and caseous glands).

3. Sudan III in oil, fed to tuberculous guinea-pigs for a short period of time (4–8 feedings) previous to death resulting from the disease, did not stain the lesions or enter the body fats to any appreciable extent.

4. Sudan III dissolved in oil and fed to tuberculous guinea-pigs did not in any way influence the progress of the disease.

5. Sudan III may be fed to guinea-pigs for a long period of time (over 200 days) in quantities of 0.05 gm. every second day, without appreciably injuring the animal.

INJECTIONS OF SUDAN III.

A sterile one per cent solution of sudan III in olive oil or butter was injected into normal guinea-pigs subcutaneously and intraperitoneally.

Experiment 11.—A guinea-pig received subcutaneously four cubic centimeters of one per cent solution of sudan III in olive oil every other day, and died on the 22d day after the first injection.

Postmortem revealed large amounts of sudan III stained oil diffused through the subcutaneous tissues with numerous small subcutaneous, gangrenous areas. The peritoneal fat was unstained, but the fluid in the peritoneal cavity had an oily appearance. The amount of oil diffused through the subcutaneous tissues and the gangrene were sufficient to account for the death of the animal.

Experiment 12.—Another guinea-pig was injected subcutaneously with sudan III olive oil every second day, and died on the 20th day.

The autopsy findings were practically the same as those observed in Experiment 10.

Experiment 13.—Another guinea-pig received four cubic centimeters of one per cent solution of sudan III in olive oil intraperitoneally every other day for 60 days, and died 93 days after the injections were begun.

Autopsy revealed about 200 c.c. of oily liquid in the peritoneal cavity, the oil being deeply stained with sudan III. The fat at the head of the testes was not stained, the mesenteric fat was pink but only superficially. Fibrinous-like masses of stained material were found around the liver and under the diaphragm. The mesenteric lymph glands were markedly enlarged and contained a large amount of sudan III. The omentum was rolled up into a mass containing fat and stain. The parenchymatous organs were not visibly changed and contained no stain.

In addition to these experiments butter was substituted for olive oil and a one per cent solution of sudan III emulsion in sterile butter was injected intraperitoneally.

Experiment 14.—A guinea-pig was injected intraperitoneally every second day with one per cent solution of sudan III in butter for 16 days, at the end of which time the animal died.

At autopsy the sudanized butter was found to a slight extent in the subcutaneous tissues at the site of injection, but mainly in the peritoneal cavity. The fatty tissues in the peritoneal cavity were only superficially stained. The omentum was deeply stained. Parenchymatous organs were not stained.

Experiment 15.—A duplicate of No. 14, lived for 15 days.

At autopsy the sudanized butter was found to be infiltrated throughout the subcutaneous and peritoneal tissues. Fatty tissues (that is, the mesenteric, post-abdominal, etc.) were not perceptibly tinted. A serofibrinous exudate was found in the peritoneal cavity, which was of deep red color as a result of impregnation with stain.

Experiment 16.—A duplicate of No. 14, lived 14 days after beginning injections.

At autopsy the small intestines were adherent in many places at the site of injection, and the peritoneal cavity contained a large amount of sudanized butter. The peritoneum was tinted pink and the fatty tissues around the uterus and tubes, kidneys and in the mesentery were superficially pink stained.

As a result of the above experiments with injections of sudan III in oil, the following conclusions seem justified:

1. After the intraperitoneal injection of sudan III dissolved in oil or in sterile butter into guinea-pigs for a period of 14 to 60 days, the sudan III remains practically unabsorbed in the peritoneal cavity where it stains superficially the body fats with which it comes in contact.

2. After subcutaneous injection into guinea-pigs of sudan III

dissolved in oil, only the fats with which the injected sudanized oil comes in contact are stained, while the other body fats (peritoneal fats, etc.) remain unstained.

A saturated (70 per cent) alcoholic solution of sudan III was injected into the testicles of guinea-pigs and was found to remain at the site of injection for a period of several days. Longer periods of time will be reported later.

Feeding Experiments with Scarlet R.

In the following feeding experiments a one per cent solution of scarlet R. in peanut oil was given to guinea-pigs.

Experiment 17.—A guinea-pig was fed six cubic centimeters of a one per cent solution of scarlet R. in peanut oil every other day for 209 days, having been inoculated with tuberculosis on the 38th day after feeding was begun, and dying of tuberculosis at the end of the feeding experiment.

Autopsy revealed a double hydrothorax and multiple areas of necrosis in the lungs, liver, and spleen which were not stained by scarlet R. Scarlet R. was, however, found in all the fat tissues, mesenteric fat, along the psoas, along the tubes and around the local enlarged caseous tuberculous glands, but the glands themselves and their caseous contents were unstained. Frozen sections of the liver, kidney, adrenals, etc., revealed no stain in the parenchymatous organs.

A guinea-pig was born on the 50th day after feeding of the mother was begun, but its body fats were not stained by scarlet R. The milk of the mother did not contain any stain, either that taken from the mammary glands or that found in the young pig's stomach. The liver of the young pig was pale and fatty, containing a large amount of unstained fat which was stainable histologically by sudan III.

Experiment 18.—A duplicate to the previous experiment was carried out, feeding scarlet R. for 166 days, the guinea-pig having been inoculated with tuberculosis on the 38th day after feeding was begun, and dying of tuberculosis at the end of the feeding experiment.

Postmortem revealed all the fatty tissues stained a dark pink (subcutaneous fatty tissues, mesenteric fat, along tubes, at the head of the kidneys, at spleen pedicle, etc.). Both lungs were solid and hard (tuberculous pneumonia) and contained a large amount of scarlet R. There was a general enlargement of lymph glands and the primary inguinal glands were large and contained a large amount of unstained caseous material. The brain and mammary glands were not stained. Ether extraction of the tissues, carried out as described in Experiment 1, resulted as follows:

Lung, ++
Spleen, +? A very faint pink tint
Kidneys, −.
Liver, +?
Fats around uterus and tubes, ++
Adrenals, −? (There may have been a bare trace of pink here, but two observers called it negative)

A pig was born to this guinea-pig on the 85th day after feeding was begun. Autopsy revealed no scarlet R. in its fat tissues, but the liver was pale and fatty, so

that histological staining with sudan III revealed a large amount of stainable fat. The milk in the stomach of this young pig was unstained, as was also that obtained from the gland of the mother pig.

Experiment 19.—A control guinea-pig, not inoculated with tuberculosis, was fed scarlet R. in peanut oil for 197 days, at which time autopsy revealed all the fat tissue stained a deep pink. The liver was normal in appearance and contained no visible stain macroscopically or microscopically on frozen section. The kidneys, lungs, spleen, brain, and cord contained no stain and were normal. There was no atrophy of body fats as was noted in the tuberculous pigs.

A guinea-pig was born 47 days after scarlet R. feeding was begun. At autopsy, shortly after birth, the body fats contained no stain, nor did the parenchymatous organs. The mesenteric lacteals stood out white and unstained in this pig. The milk from the mother and that found in the stomach of the young pig was unstained.

As a result of the above feeding experiments with scarlet R. in guinea-pigs the following conclusions seem justified:

1. Scarlet R. fed to guinea-pigs, normal and tuberculous, for a long period of time (166–209 days), stains all the body fats pink, but does not stain the parenchymatous organs.

2. Scarlet R. in oil fed to tuberculous guinea-pigs previous to and during the disease does not stain the tuberculous lesion or noticeably affect the progress of the disease.

3. Scarlet R. in oil fed to guinea-pigs for a long period of time (47–85 days), does not stain any of the tissues of the young or pass into the milk of the adult guinea-pig.

4. The feeding to the mother of a large amount of oil stained with scarlet R. produces a fatty liver in the unborn young.

EXPERIMENTS WITH INJECTIONS OF NILE BLUE SULFATE.

This is a water-soluble fat dye which possesses the added advantage of staining the neutral fats and fatty acids differently from one another.[1]

Subcutaneous and intraperitoneal injections in physiological salt solution were made in the following experiments:

Experiment 20.—A guinea-pig was injected subcutaneously on alternate days with three cubic centimeters of two per cent Nile blue sulfate in physiological salt solution in three different places; a week after the first injection the skin and surrounding tissues became necrotic and sloughed out.

Experiment 21.—A second guinea-pig, duplicate of 20, developed the same necrosis, and sloughing of the skin.

[1] For literature on Nile blue sulfate see J. Lorrain Smith, *Jour. Path. and Bact.*, 1907, 12, p. 1; and Holthusen, *Zieglers Beitr.*, 1910, 49, p. 595.

Experiment 22.—A guinea-pig was given intraperitoneally three injections of three cubic centimeters two per cent Nile blue sulfate in physiological salt solution on alternate days, and was found dead on the eighth day.

Postmortem revealed a large amount of fibrinous exudate in the peritoneal cavity, which contained a blue stained fluid. The abdominal viscera were speckled with particles of stain, but not uniformly stained. The spleen and kidneys were not stained, but simply covered with a fibrinous exudate containing blue particles; the liver was also unstained. The lymphatic vessels of the anterior thoracic wall were slightly blue tinted. Heart, lungs, adrenals, brain, and cord were unstained.

Experiment 23.—A guinea-pig treated exactly as 22 was also dead in eight days. The stain was mainly found in the fibrinous exudate in the peritoneal cavity, the lymphatics in the anterior thoracic wall not being stained in this animal.

FEEDING EXPERIMENTS WITH NILE BLUE SULFATE.

Feeding experiments with Nile blue sulfate were carried out, using a one per cent emulsion in peanut oil. Attempts were made to feed the dye in aqueous solution, but without success because the guinea-pigs reject water solutions of the dyes.

Experiment 24.—A guinea-pig was given on alternate days five cubic centimeters of one per cent solution of Nile blue sulfate in peanut oil for 151 days, having been inoculated with tuberculosis on the 38th day after feeding was begun. At the end of the feeding period, the stain was not found anywhere in the body of the animal, but the animal had numerous large necrotic areas in the liver and spleen, and the lymph glands of the body were enlarged, the left inguinal glands containing a large amount of caseous material unstained. After a general rapid inspection of the animal a forced aortic injection of one per cent Nile blue sulfate in 0.9 per cent NaCl solution containing a small amount of hydrogen peroxid was made. The stain rapidly passed through the tissues, but did not stain the necrotic areas or the fats to any extent. In the liver and spleen a few nondescript nuclei were stained, otherwise the experiment was negative.

Experiment 25.—A guinea-pig (duplicate of 24) was fed one per cent solution of Nile blue sulfate in peanut oil for 149 days, having been inoculated with tuberculosis on the 38th day and dying of tuberculosis at the end of the feeding period.

Postmortem revealed numerous necrotic areas in the spleen and liver unstained. The local inguinal glands were caseous and unstained. The body fat was atrophied but unstained. The kidneys, adrenals, lungs, etc., were unstained. The tissues treated with H_2O_2 did not take on any blue color.

Experiment 26.—A normal guinea-pig was fed one per cent solution of Nile blue sulfate in peanut oil for 221 days, at which time it was still in good health. Killed with ether.

Autopsy revealed no stain (macroscopically or microscopically) in the fat tissues of the body or in any of the parenchymatous organs.

A guinea-pig was born 103 days after feeding was begun but contained no stain, either in the fat tissues or parenchymatous organs. The milk from the mother and that found in the young pig's stomach was not stained.

Another guinea-pig was born 187 days after beginning feeding.

Postmortem revealed no stain anywhere in the young pig. The milk again was negative for stain. The liver of the young pig was pale and fatty. (Sudan III staining revealed a large amount of fat in the liver.)

A second pig born 187 days after feeding began was allowed to live eight days, at the end of which time no stain was found in the body. The liver, however, was still pale, but not so much so as that in the previous young pig.

As a result of the above experiments with Nile blue sulfate the following conclusions seem justified:

1. Nile blue sulfate in two per cent solution in 0.9 per cent NaCl is toxic when injected intraperitoneally, causing a marked serofibrinous peritoneal exudate, and death after three injections of three cubic centimeters on alternate days. Subcutaneously injected, it causes necrosis of the tissues and sloughing at the site of injection, the stain itself not staining any of the tissues but rather remaining at the site of injection.

2. Although highly toxic when injected, Nile blue sulfate seems to have no toxic effect when fed in oil for long periods of time.

3. Feeding of Nile blue sulfate in oil emulsion did not lead to staining of the fats or parenchymatous organs of the guinea-pig.

4. Feeding Nile blue sulfate in oil emulsion to guinea-pigs infected with tuberculosis after feeding had begun did not result in staining the tuberculous lesions (caseous lymph glands and necrotic areas in the spleen and liver) or visibly influence the progress of the disease.

FEEDING EXPERIMENTS WITH SUDAN YELLOW (META-DIOXYAZOBENZOL).

Four guinea-pigs were fed a one per cent solution of sudan yellow in peanut oil, two of these were inoculated with tuberculosis and two remained non-infected throughout the entire period of the experiment.

Experiment 27.—A normal guinea-pig was fed sudan yellow on alternate days for 144 days, at the end of which time postmortem revealed a normal liver (not fatty), lungs, kidneys, adrenals, brain, etc., containing no yellow stain visible macroscopically or microscopically. The fats of the body were not more yellow than found in normal unfed animals. The urine in the bladder was a distinct yellow, however, whereas frozen sections of the kidney did not contain any of the yellow dye.

Experiment 28.—A second normal guinea-pig was fed sudan yellow for 104 days and the postmortem findings were the same as those observed in the previous pig. No macroscopic stain was observed in any of the tissues of the animal, but the bladder was filled with a deep yellow urine, and frozen sections of the kidney appeared to contain a little yellow pigment, especially in the glomeruli.

Experiment 29.—A guinea-pig was fed one per cent solution of sudan yellow in peanut oil every other day for 97 days, having been inoculated with tuberculosis on the 33d day of the feeding.

Autopsy at the end of the feeding period revealed necrotic areas in the liver, spleen, and a few in the kidneys. The necrotic areas in the liver and spleen were of a greenish yellow color, but the yellow was not due to the dye as the same yellowish coloration had been noticed in animals with tuberculosis not receiving any dyes, and as shaking out the tissue with ether revealed no yellow coloration of the ether layer beyond that ordinarily obtained from liver tissue. The local caseous inguinal glands were unstained. The fat tissues were atrophied but contained no dye. The urine in the bladder was a deep yellow color, but the kidney was not stained.

A guinea-pig was born on the 88th day after feeding was begun, but it contained none of the yellow dye. The milk of the mother pig was not stained yellow.

Experiment 30.—A second guinea-pig was fed sudan yellow as in preceding experiment for 145 days, having been inoculated with tuberculosis on the 33d day after feeding was begun.

Autopsy at the end of the feeding period revealed enlarged caseous local inguinal glands (unstained) and enlargement of the lymph glands generally (unstained), numerous necrotic areas in the liver of greenish yellow color, the spleen was much enlarged and contained large, white necrotic areas. The fat tissues of the body (at the head of the testes, posterior abdominal, and at the back of the neck) were stained a faint pale yellow color. The brain and cord were unstained. There was no urine in the bladder and the bladder itself was not stained yellow.

The above experiments in which sudan yellow was fed to normal and tuberculous guinea-pigs seem to justify the following conclusions:

1. Sudan yellow fed to normal and tuberculous guinea-pigs does not stain the organs of that animal nor to an appreciable degree, the body fats within 145 days, but it does color the urine yellow.

2. Sudan yellow fed to an adult guinea-pig does not color the milk of the mother or the young guinea-pig, even when fed for a long period of time (88 days).

3. Sudan yellow fed to guinea-pigs infected with tuberculosis after feeding was begun does not enter the tuberculous lesions (caseous lymph glands or necrotic areas in the liver and spleen).

FEEDING EXPERIMENTS WITH SUDAN BROWN (α-OXYAZONAPHTHALIN).

Normal and tuberculous guinea-pigs were fed sudan brown in peanut oil for long periods of time to see whether *intra-vitam* staining with this fat stain was possible.

Experiment 31.—A normal guinea-pig was fed four to five cubic centimeters of a one per cent solution of sudan brown in peanut oil on alternate days for 144 days, at the end of which time it was killed. Postmortem examination revealed a faint brown

tint of the fat tissues of the body, but no macroscopic or microscopic staining of any parenchymatous organs. The liver, spleen, kidneys, adrenals, and brain were normal and unstained. Bladder contained unstained urine. In the uterus were found two half-developed embryos, unstained. The placentas were unstained.

A guinea-pig, born 94 days after feeding was begun, contained no stain, either in the fat tissues or in the parenchymatous tissues. The milk of the adult guinea-pig was unstained.

Experiment 32.—A guinea-pig was fed four to five cubic centimeters of a one per cent solution of sudan brown in peanut oil on alternate days for 115 days, having been inoculated with tuberculosis on the 33d day after feeding was begun, and dying of tuberculosis at the end of the feeding period.

Autopsy revealed practically no visible stain in the fat tissues. The local inguinal glands were enlarged and caseous, but were not stained. The liver and spleen contained numerous large necrotic areas which were pale yellow and white (unstained). The adrenals, kidneys, brain, etc., were unstained macroscopically and microscopically.

Experiment 33.—A second guinea-pig was fed sudan brown on alternate days for 145 days, having been inoculated with tuberculosis on the 33d day. At the end of the feeding period autopsy revealed a faint brown tint in the fat tissues of the body (especially at the back of the neck and around the tubes). The liver, spleen, and lung contained numerous white and yellow necrotic areas, unstained. The local inguinal glands were enlarged and caseous, but unstained. The kidneys, adrenals, bladder, urine, brain, and cord were unstained.

As a result of the above feeding experiments with sudan brown, the following conclusions seem justified:

1. Sudan brown when fed, stains the body fats of normal and tuberculous guinea-pigs only slightly, if at all, within 145 days. It does not stain any of the parenchymatous organs.

2. In tuberculous animals sudan brown, fed for a long period of time, does not stain the tuberculous lesions (caseating lymph glands and necrotic areas in the liver, spleen, or lungs).

FEEDING EXPERIMENTS WITH EOSIN A.

A series of four guinea-pigs were fed Eosin A, water-soluble, in suspension in peanut oil for periods of 100 to 133 days. Two of the pigs remained normal throughout the feeding period, and two of them were inoculated with tuberculosis on the 21st day after feeding was begun. The results obtained with these animals were entirely negative, the dye was not found in any of the parenchymatous organs or tissues, so that absorption apparently had not taken place by this method of administration. The tuberculous lesions (local caseous lymph glands, necrotic areas in the lungs, liver, and spleen) also were unstained.

DISCUSSION OF RESULTS.

These experiments carry over to the lesions of tuberculosis the results obtained by the biologists who have investigated the behavior of sudan III and other fat stains under normal and some

pathological conditions. Their results indicate that the fat dye which enters the body dissolved in fat remains either entirely or chiefly with this same food fat, being deposited with it if the food fat is deposited, but not leaving the food fat to enter either stored fat or the intracellular fats and lipoids of active tissues. *The fats of tubercles never show any of the administered fat dyes*, no matter whether the tubercles formed before or after the animal was saturated with the dye. Therefore, it seems probable that the fats microscopically visible or chemically demonstrable in tubercles, are derived chiefly or solely from the existing fats and lipoids of the disintegrated cells, not by deposition from the fats in the blood, a view entirely in harmony with the histological evidence. Attention may also be called to the absence of any visible staining of the tubercle bacilli within the lesions by the administered fat dyes. This result is to be expected in view of the very limited extent to which tubercle bacilli are stainable with fat dyes even *in vitro*, as shown by other experiments performed in this laboratory, which will soon be reported.

The fat dyes seem to be almost entirely innocuous to guinea-pigs, when given by mouth, as observed by other experimenters. In view of the striking effect of fat dyes upon cell proliferation as shown by B. Fischer, this apparent inertness of the same dyes when fed to animals is rather surprising. Even with tuberculous animals the thorough saturation of the animal with the dye has not seemed to lower the resistance to the disease. Female guinea-pigs, thoroughly saturated with these dyes, give birth to sound offspring which show on autopsy no abnormalities other than a fatty liver. The observation of the Gages that the fat dyes do not pass through the placenta to the fetus is confirmed by these experiments, as also by Mendel and Daniels. This fact would seem to be of great importance in general biology, for it seems to indicate that the fats of the fetus are not derived as such from the mother but are formed, either partly or wholly, in the body of the fetus itself. In contrast with this is the fact that in birds and reptiles the fat stain enters the ovum very quickly and abundantly, and during development passes into the fats of the embryo. It is difficult to believe that between birds and mammals there exists so

fundamental a difference in developmental chemistry as these observations imply, and more extensive observations along this line are needed.

The failure of fat dyes to enter brain and cord, as observed by others, is corroborated and extended to other dyes. Jacobsthal says that scarlet R. enters the adrenals, but no positive results were obtained in the animals of this series. Neither was any staining of the milk of lactating female guinea-pigs ever observed, although described in the rat by the Gages; and in cats, rats, goats and to a very slight extent in the guinea-pig by Mendel and Daniels. The reason for these discrepancies is not apparent.

SUMMARY.

Fat dyes administered under various conditions to guinea-pigs with tuberculosis never were found within the tubercles. This is in favor of the view that the fats of the tubercle are derived from the intracellular fats of the tissues forming the tubercle, and not from food fats or transported depot fats.

Tubercle bacilli within tubercles in such animals do not become stained with the fat stains.

Saturation of the animal with common fat dyes has no noticeable influence on the course of a tuberculous infection in the guinea-pig.

The fat stains employed in these experiments (sudan III, scarlet R., sudan yellow, sudan brown, Nile blue sulfate) were never found to stain any but depot fats, not being present in the parenchymatous cells whether normal or diseased. They caused no apparent harm to the guinea-pigs even after being fed to them over 200 days.

Subcutaneous or intraperitoneal injection of fat dyes dissolved in fats and oils is much less effective in staining the depot fats, than is feeding the stained fats.

Nile blue sulfate is toxic when given by injection, and when fed it did not cause staining of the fat tissues. Sudan yellow also failed to stain the fat tissues, and is excreted by the kidneys. Sudan brown has little or no effect on the fats. Scarlet R. gave a more intense coloration than sudan III but in general the effects are about the same.

Fat dyes do not pass through the guinea-pig placenta to the

fetus, or at least the fat of embryo pigs from stained mothers is not stained (corroborating the results of the Gages, Mendel, and Daniels). The embryos often show extremely fatty but unstained livers. Fat dyes were never observed to pass into the milk of lactating guinea-pigs, although positive results have been obtained by others.

THE LIPASE OF BACILLUS TUBERCULOSIS AND OTHER BACTERIA.*

STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS. III.

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The study of the fats and lipoids of bacteria calls attention to the presence of enzymes within the bacteria which may act upon substances of this class. That living, growing bacteria cause splitting of fats is shown beyond question by the decomposition of fats and butter by bacteria, but the particulars of the process seem to have received but little attention. The chief work on this subject was done by Eijkman,[1] who devised a simple plate method for studying the effects of various bacteria upon different substances, among them the higher fats. According to the evidence of this method, which is of necessity crude and gives only gross results, beef tallow is split into fatty acids and glycerin in the vicinity of colonies of *B. pyocyaneus*, *Staphylococcus pyogenes aureus*, *B. fluorescens* and *B. prodigiosus;* but not by colonies of *B. anthracis*, *coli communis*, *typhosus*, *diphtheriae*, *mallei*, *pestis*, *dysenteriae* (Kruse), or by cholera vibrios and several common saprophytes. More recently this work has been extended by Söhngen who used the original "auxanographic" method of Eijkman and a modification of the same.[2] To the foregoing list of lipolytic bacteria he adds a few more forms, all non-pathogenic. According to his observations several of the fat-splitting bacteria seem to produce two lipases. One of these, α-lipase, diffuses more rapidly and splits fat in either acid or alkaline medium; the other, β-lipase, is formed in an acid medium but does not split fats in it. H-ions inhibit and OH-ions increase the decomposition of fats in culture, and $0.02N$ acid completely inhibits lipolysis. Bacterial lipases show a great

* Received for publication August 24, 1912.

[1] *Centralbl. f. Bakt.*, I. Abt., 1901, 29, p. 841.

[2] *Kon. Akad. Wetenschappen*, 1911, 13^2, pp. 667 and 1200.

similarity in behavior to pancreas and liver lipase, and he found them capable of synthesizing fat from oleic acid and glycerol, the monoglyceride being chiefly formed. In *B. pyocyaneus*, *fluorescens*, and *liquifaciens* there is a lipase which resists heating to 100° C. for five minutes.[1] In a few of these experiments, Söhngen used extracts or killed cultures of bacteria, showing that lipases actually exist in the cultures.

Beyond the above studies by the Dutch bacteriologists there is, so far as we can find, little else in the literature bearing upon the presence of lipase in bacteria. Fuhrmann, in his *Vorlesungen über Bakterienenzyme*, gives a few references to examples of the splitting of fats by living bacteria, e.g., making butter rancid. In most of these experiments the results merely demonstrate that during bacterial growth fats may be split, but, with the exception of a few of Söhngen's experiments, do not show whether this is the result of a true lipase or some other agency, e.g., alkalies. Schreiber,[2] indeed, did try to demonstrate the presence of lipase by growing mass cultures of a fat-splitting bacillus in peptone water, and using this culture in 1-1000 thymol water. This killed culture exerted no splitting effect on almond oil. Fuhrmann objects to this experiment on the ground that in molds and other higher fungi there are lipases which can only be demonstrated after the cells have been shattered; but under the conditions of the experiment there should be enough disintegration of the cells by autolysis to liberate any existing lipase. We should object more to the choice of almond oil as an indicator of lipase action, since such insoluble oils offer so little surface for attack that lipolysis is very slow and of little extent, and the resulting free fatty acid is difficult of demonstration by titration in aqueous solution. As for the presence of lipase in *B. tuberculosis*, there is almost nothing known. In 1901 Carrière[3] reported that broth cultures of the tubercle bacillus cause acid formation in monobutyrin. The action is destroyed by heat, but not by chloroform or ammonium fluoride, hence he concludes that it must be due to a true lipase, or a monobutyrase similar to that found by Hanriot in animal tissues. While this conclusion

[1] Söhngen, *ibid.*, 1911, 20, p. 126.

[2] *Arch. f. Hyg.*, 1902, 41, p. 328.

[3] *Compt. rend. Soc. de Biol.*, 1901, 53, p. 320.

is probably correct, yet the evidence is unconvincing, since the titrations were performed with sodium carbonate, with phenolphthalin as indicator, so that at least the quantitative value of the results is nothing. Also, monobutyrin is not a conclusive indicator for the presence of typical fat-splitting enzymes.

Further than the above brief report we can find no statements concerning the lipolytic activity of *B. tuberculosis*. Since this organism is characterized by its power of forming and storing within itself fatty substances, and since, as previously explained, the fats of the tubercle bacillus seem of great importance in the chemotherapy of tuberculosis, we have investigated the lipolytic and ester-splitting capacity of this organism and compared it with other bacteria.

Attempts to determine the ability of tubercle bacilli to cause splitting of tallow in plate cultures, according to Eijkman's method, were unsuccessful. As this method is, at the best, not proof of the existence of lipase in bacterial cells, we did not continue these experiments exhaustively, especially when we found that organisms giving negative results by the plate method may yet possess considerable lipolytic power. Direct experiments were performed as follows:

Large quantities of the bacteria to be tested were grown on agar in flat bottles, the growth from 40 to 80 such bottles, each with a surface of about 15×5.5 cm. being used in each experiment. After a maximum growth had been reached, the bacteria were scraped off into toluene water, or washed off with toluene water, and kept at room temperature for 24 hours or longer, with an excess of toluene, and thoroughly agitated at intervals. This killed all the bacteria, as was shown by the cultivation experiments, summarized in Table 1, and also allowed a certain amount of autolysis to permit of the liberation of endocellular enzymes.

These tests show that none of the nonsporulated bacteria can survive 24 hours in toluene water, and certainly guarantee the absence of any active growth in such a medium after that period. Samples of the emulsion of tubercle bacilli used in our experiments were injected into guinea-pigs in doses containing 0.02 and 0.13 mg. of bacilli which had stood in the toluene water 20 hours and five days. None of the animals developed tuberculosis from these large doses, showing that in our experiments we were dealing with dead bacilli only.

Samples of the emulsions were taken and the dry weight of the contained bacteria determined. The emulsions were then divided into equal samples; one of each pair being heated for one-half hour submerged in boiling water to destroy all thermolabile enzymes and thus serve as a control. To each pair of samples was then added a portion of the ester to be tested, olive oil, ethyl-butyrate, or triacetin (glycerol triacetate), a slight excess of toluene, and a few drops of phenolphthalin solution; the mixture was

made neutral with $n/10$ NaOH, tightly stoppered and put in the incubator. At intervals the mixture was brought to the neutral point, and in the tables below are given the amount of $n/10$ alkali in cubic centimeters required for this purpose. In the case of actively lipolytic bacteria, part of the acidity was neutralized by $n/1$ alkali to prevent excessive dilution.

TABLE 1.

GERMICIDAL ACTION OF TOLUENE AT INCUBATOR TEMPERATURE.

TIME	*B. Prodigiosus*			*B. Subtilis*			*B. Coli*			*Staph. Aureus*			*B. Pyocyaneus*		
	Selenium* 1:50,000	Broth	Plain Agar	Selenium	Broth	Agar	Selenium	Broth	Agar	Selenium	Broth	Agar	Selenium	Broth	Agar
10 minutes at room temperature	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−
30 minutes incubation	−	−	−	+	+	+	+?	−	+	+	+	+	−	+?	−
2 hours incubation	−	−	−	+?	+	+	−	−	−	+	+	+	−	−	−
6 hours incubation	−	−	−	+	+	+	+?	−	−	+?	+?	+	−	−	.
24 hours incubation	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−
48 hours incubation	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−
4 days incubation	..	..	..	+	+	+	..	..	..	..	..	..	..	..	..
10 days incubation	..	..	..	+?	+	+	..	..	..	..	..	..	..	..	..

* Selenium in a dilution of 1:50,000 was used in broth as an index of the presence of living bacteria, which cause reduction (Gosio, *Ztschr. f. Hyg.*, 1905, 51, p. 65).

Samples of each emulsion were dried and weighed, the solid substance, consisting almost entirely of bacterial cells being, in each experiment, as follows: *B. coli*, 0.945 gm.; *B. dysenteriae* (Flexner), 0.81 gm.; *Staph. pyogenes aureus*, 1.140 gm.; *B. pyocyaneus*, 0.75 gm.; with *B. tuberculosis*, there was used in each experiment two grams of fresh bacilli or about 0.22 gm. dry weight of bacilli, washed free from all other substances.

TABLE 2.

OLIVE OIL, 4 C.C.

	2 Days	5 Days	8 Days	10 Days	14 Days	Ether Extract*	Total
B. coli, heated	0.0	0 0	0.0	0 0	0.0	0.73	0 73
" " fresh	2.50	1.82	1.55	0.50	1.72	9.45	15 54
B. dysenteriae, heated	0.0	0 0	0.0	0 0	0.0	0 60	0 60
" " fresh	2.18	0 80	0.92	0.0	0.50	3.20	7 60
B. pyocyaneus, heated	0.0	0.0	0 0	0.0	0.0	1.42	1.42
" " fresh	24.46	14 50	4.15	3 90	2 35	18.86	68.22
" " fresh, no oil	1.70	1.90	1.40	1.10	1 51	....	7 61
Staph. pyogenes aureus, heated	0.0	0.0	0.0	0.0	0 0	0.45	0.45
" " " fresh	4.65	2.0	0.0	0.0	0 0	17 70	24.35
B. tuberculosis, heated	0.0	0.0	0.0	0.0	0 10	0 88	0.98
" " fresh	0.0	0.0	0.0	0 0	0.0	2 68	2.68
Water and toluene	0.0	0.0	0.0	0 42	0.10	0 94	1 46

* On account of the tendency of the olive oil suspensions to form soapy emulsions which would not titrate well, and because the fatty acids liberated are held in the emulsion in such a way that the watery alkaline solution does not reach them readily, the titrations in the aqueous suspensions are not at all accurate. After the 14 days incubation the emulsions were shaken out with neutralized ether, and the fatty acids titrated with alcoholic NaOH and HCL, the resulting figure giving the amount of $n/10$ alcoholic NaOH solution required to neutralize.

TABLE 3.

ETHYL BUTYRATE, 8 C.C.

	2 Days	5 Days	8 Days	10 Days	14 Days	Total
B. coli, heated	2.80	3.02	2.75	2.80	3.08	14.45
" " fresh	13.00	13.12	11.35	9.88	10.62	57.97
B. dysenteriae, heated	2.97	3.58	4.10	4.42	4.80	19.87
" " fresh	16.20	15.91	17.60	24.43	24.30	98.44
Staph. pyogenes aureus, heated	4.85	6.15	6.15	6.30	4.00	27.45
" " " fresh	122.80	62.85	38.82	35.00	34.50	293.97
B. pyocyaneus, heated	3.20	4.20	3.65	3.60	4.00	18.65
" " fresh	52.28	78.40	62.85	51.00	43.90	288.43
B. tuberculosis, heated	0.81	1.27	1.08	0.89	1.10	5.05
" " fresh	8.92	4.26	2.30	2.46	2.44	20.38
Water and toluene	0.60	0.70	0.80	0.74	0.78	3.62

TABLE 4.

TRIACETIN, 4 C.C.

	2 Days	5 Days	8 Days	10 Days	14 Days	Total
B. coli, heated	6.00	7.85	6.80	6.50	8.0	35.15
" " fresh	19.50	19.25	15.35	13.98	15.18	83.26
B. dysenteriae, heated	4.20	4.95	6.25	7.60	5.90	28.90
" " fresh	27.75	29.25	30.17	47.50	37.15	171.82
Staph. pyogenes aureus heated	7.15	8.35	9.32	10.80	9.00	44.62
" " " fresh	142.35	182.00	70.00	60.00	25.00	479.35
B. pyocyaneus, heated	7.50	10.20	7.90	8.30	9.50	43.40
" " fresh	73.00	77.20	53.00	26.50	25.00	254.70
B. tuberculosis, heated	1.83	1.97	2.31	3.16	4.62	13.89
" " fresh	10.55	9.88	9.03	11.49	20.35	61.30
Water and toluene	1.45	2.07	2.11	2.40	4.88	12.91

Inspection of these tables shows at once a definite hydrolytic activity on the part of all the bacteria studied. While *B. pyocyaneus* and *Staphylococcus pyogenes aureus*, which are classed by Eijkman as among the fat-splitting bacteria according to the plate method, show by far the most active lipolysis, yet *B. dysenteriae* and *B. coli*, which do not split tallow under agar plate colonies, are possessed of no inconsiderable lipolytic activity. From this it seems safe to conclude that the plate method as heretofore used shows only the most active degrees of lipolysis, and is solely a relative test for lipase. Bacteria not giving positive results by this method may be, nevertheless, possessed of considerable lipolytic activity. It is, indeed, highly improbable that any living cells are

devoid of lipolytic activity, whether animal, plant, or bacterial cells. A possible source of error might be imagined if bacteria undergoing autolysis produced acids from other sources than fats, e.g., lactic acid from carbohydrates. This possibility is eliminated by the experiments with olive oil, which show that almost no water-soluble acid is produced by these bacterial suspensions during autolysis except by *B. pyocyaneus*, and here the amount is relatively very insignificant.

It is noticeable that a certain amount of hydrolysis is shown by the cultures which have been heated for 30 minutes at approximately 100° C.; also, that this varies in a measure in direct proportion to the hydrolytic activity of the unheated killed bacteria, as if it represented a residual portion of the thermolabile enzyme. Without attempting to analyze further or to interpret this observation, it may be compared with the statement of Söhngen that certain bacteria possess a lipase resisting 100° C. for five minutes, which he interprets as a special thermostabile lipase.

As for *B. tuberculosis* it is apparent that, in common with all its biological properties, it is not an active splitter of fats and simpler esters; but these experiments do demonstrate that it does possess enzymes capable of slowly hydrolyzing the three very different esters used in these experiments. However, it probably falls short in activity of *B. coli* and *B. dysenteriae*, which are among the least actively lipolytic of the rapidly growing pathogenic bacteria, but presumably the low figures are at least partly ascribable to the use of smaller quantities of bacillary substance. There are too many complicating factors in the way of bacterial fats, adventitious substances, etc., in ascertaining the quantity of bacterial substance used to make our results of much quantitative value.

One noticeable fact is that each bacterium exerts a corresponding effect on each of the three sets of esters used, the staphylococcus being most active in hydrolyzing each, pyocyaneus coming second, colon, dysentery, and tubercle bacilli following in order. This suggests that the same enzyme is concerned in each case, which seems more probable than the assumption that each ester is hydrolyzed by a specific enzyme and that all three enzymes are developed to a corresponding degree in each of the five species.

Kastle, Loevenhart, Amberg, and Peirce[1] have directed attention to the extreme susceptibility of the lipases of animal tissues to fluorides, so that marked inhibition of ester splitting is caused by dilutions of NaF as high as 1-500,000 or more. This inhibition is most marked with esters of the lower fatty acids, decreasing as one ascends in the fatty acid series of esters. Experiments were, therefore, performed to discover whether bacterial lipases are similar to animal tissue lipases in this respect.

In the first series, the growth on 80 bottles of a 48-hour culture of *B. pyocyaneus* was suspended in 420 c.c. toluene water, and the dried weight of bacterial substance present in 10 c.c. samples was found to be 0.203 gm. Samples of 50 c.c., containing each 1.015 gm. of bacterial substance, were then used for the experiments, to each being added sufficient 1-1000 NaF solution to make the strengths indicated, the total amount of fluid being made approximately the same in each sample. Because of the large amount of acid developed, the titration was first made approximately with normal alkali, to avoid too great dilution and then finished with $n/10$ solutions, the figures given being for $n/10$ NaOH. After each titration, sufficient NaF solution was added to hold the fluoride concentration at approximately the given figure. In order to ascertain the influence of the amount of enzyme on these results, in the second experiment but 10 bottles of culture were used, giving 0.145 gm. of bacterial substance for each sample. Here the titrations were made with $n/4$ solutions, but the figures are given for $n/10$ alkali as in all the other experiments.

These experiments indicate that the lipase of *B. pyocyaneus* behaves under the influence of sodium fluoride exactly as Loevenhart and his co-workers found the lipase of liver and pancreas to behave. In Series A during the first three days there is a decided inhibition of the hydrolysis, but after this time the degree of inhibition becomes less and less marked, until at the end of two weeks there may be even more rapid hydrolysis in the presence of the fluoride. Series B, in which was used a much smaller amount of bacterial substance, shows a similar tendency, which appears later.

[1] Loevenhart and Peirce, *Jour. Biol. Chem.*, 1907, 2, p. 397; and Amberg and Loevenhart, *ibid.*, 1908, 4, p. 149.

This behavior agrees perfectly with the findings of the above authors, and is ascribable to a number of factors. One is the accumulation in the solution of the sodium salt of the acid formed by hydrolysis, which Amberg and Loevenhart found to counteract the effects of the fluoride. Another is their observation that the fluoride causes final equilibrium to be reached more slowly, so that after the first rapid hydrolysis without the presence of fluoride

TABLE 5.

EXPERIMENT A. 1.015 GM. *B. Pyocyaneus.*

	1 Day	3 Days	6 Days	10 Days	14 Days	17 Days
Triacetin—						
NaF, 1-3,000	46.9	68.7	73.0	60.0	37.2	30.8
NaF, 1-37,500	69.1	97.2	85.0	62.0	37.5	29.6
NaF, 1-150,000	83.5	117.4	79.0	62.0	39.5	29.8
Control	90.4	119.5	74.0	59.5	35.5	28.4
Ethyl butyrate—						
NaF, 1-3,000	12.8	18.5	15.4	10.7	14.6	10.7
NaF, 1-37,500	16.4	14.0	15.0	11.5	13.8	10.9
NaF, 1-150,000	16.5	23.5	18.7	8.3	8.0	8.8
Control	17.0	20.0	16.0	8.3	9.8	8.2

EXPERIMENT B. 0.145 GM. *B. Pyocyaneus.*

Triacetin—						
NaF, 1-3,000	11.0	16.2	19.6	33.4	33.6	40.3
NaF, 1-37,500	13.4	24.1	34.5	46.4	44.0	56.1
NaF, 1-150,000	19.0	30.0	47.3	53.7	41.5	57.0
Control	22.3	38.8	48.8	56.0	43.8	51.2

the digest which has been held back by the fluoride may then show more active hydrolysis during a given period of time. Also, the accumulation of acid in the digest injures the lipase, and the reduction in acid formation by fluorides will also decrease the injury to the lipase from this source and tend to keep it active for a longer period. The observations of Loevenhart and Peirce, that hydrolysis of triacetin by animal lipase is more depressed by fluorides than the hydrolysis of ethyl butyrate, and also that the larger the amount of enzyme the less the effect of the fluoride, are also duplicated by pyocyaneus lipase. It would seem that the similarity of the results is so great as to warrant the conclusion that the lipase of *B. pyocyaneus* is of the same character as the lipase of mammalian liver and pancreas.

SUMMARY.

By directly testing the killed bacterial substance upon various esters and fats, the presence in bacteria of typical lipolytic enzymes can be demonstrated, even with organisms which cause no visible splitting of fats in plate cultures (the "auxanographic" method). Bacteria causing visible fat splitting in plate cultures (*B. pyocyaneus* and *Staphylococcus pyogenes aureus*) are much more actively lipolytic than bacteria which do not give positive results by this method (*B. coli communis*, *B. dysenteriae* [Flexner], *B. tuberculosis*). All these five organisms cause hydrolysis of olive oil, ethyl butyrate, and glycerol-triacetate. The order of hydrolytic activity is the same for each of the three esters, being as follows: *Staphylococcus*, *B. pyocyaneus*, *B. coli*, *B. dysenteriae*, and *B. tuberculosis*. Presumably the same enzyme attacks all three esters. *B. tuberculosis* is probably less actively lipolytic than the other bacteria tested.

The bacterial lipases resemble the lipases of mammalian tissues in being inhibited by sodium fluoride in high dilutions. The similarity of behavior is so great as to indicate the similarity or identity of the lipases of bacteria and mammalian tissues.

THE PRODUCTION OF ANTIRABIC IMMUNITY BY INTRA-SPINAL INJECTIONS OF VIRUS.*†

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The object of this paper is to present the results of an attempt to produce a rapid antirabic immunity by direct immunization of the brain cells.

The method devised by Pasteur for the prevention of hydrophobia limited this work to large and well equipped laboratories, and required that the patient undergo treatment for a period of three weeks or more. Several modifications have been adopted from time to time which have made the work of preparing the material less onerous, and the treatment more available to the public, especially to those patients living at a distance from such a laboratory. In all antirabic institutes, whatever modification may have been adopted in the preparation of the material, the treatment consists in a number of injections into the abdominal wall. The result of this is an indirect immunization of the brain cells against the infective agent. In spite of all treatment, however, a small number of the patients die before immunity can be established.

During the past year I have described a method[1] for preserving the virus used in antirabic immunization. This, in brief, consists in freezing with carbon dioxid snow the brain and cord of a rabbit infected with the disease, and drying these in a vacuum at a temperature of $-10°$ C. Material prepared in this manner and kept free from moisture in an ice box (10° C.) loses its infectivity so slowly that after a preservation of 40 weeks one-tenth of a milligram when injected subdurally will produce paresis in a rabbit in seven days. In other words, after eight months of desiccation its infectivity is equal to that of Pasteur's two-day cord.

* Received for publication October 4, 1912.

† Read before the International Congress on Hygiene and Demography, Washington, D.C., September 24, 1912.

[1] *Jour. Infect. Dis.*, 1912, 10, p. 369.

In testing the desiccated material from time to time to determine the effect of time, temperature, and light on the rate of loss of infectivity, I observed that some rabbits which had received subdurally a quantity of the virus slightly less than the minimal infective dose developed an appreciable and determinable immunity against subsequent subdural inoculations.

Ten rabbits were immunized in this manner and the degree of immunity thus acquired was ascertained by one or more later tests. Of these ten, six resisted an injection sufficient to kill untreated controls. One did not develop the disease after an injection of twice the minimal infective dose. One survived the injection of 100 M.I.D. Two died on the 15th and 17th days, respectively, after the injection of twice the minimal infective dose.

Of seven rabbits that survived the first test, four were inoculated later with 10 M.I.D. of street virus. Two survived and two died on the 18th day. All injections were made under the dura after trephining. Table 1 gives the results of these experiments in detail.

TABLE 1.

Rabbit	Amount of First Inoculation	Time of Second Inoculation	Amount	Time of Third Inoculation	Amount	Result
1	½ M.I.D.	30 days	2 M.I.D.	....	....	Died 15th day
2	⅙ "	58 "	2 "	....	....	" 17th "
3	⅔ "	29 "	2 "	....	....	Immune
4	½ "	43 "	1 "	72 days	10 M.I.D. s.v.	Died
5	½ "	43 "	1 "	72 "	10 " "	"
6	⅓ "	27 "	1 "	78 "	10 " "	Immune
7	¼ "	27 "	1 "	107 "	10 " "	"
8	½ "	78 "	1 "	107 "	100 " "	Died
9	$\frac{1}{10}$ mg.	27 "	1 "	107 "	200 " "	"
10	¼ "	36 "	100 "	55 "	200 " "	"

Tests made in one lot of material which had been kept for some months at room temperature in daylight showed this to be non-infective, and it was observed that the rabbit inoculated with this material had also acquired an immunity (Table 1, rabbit No. 9).

Following this, a number of rabbits were given larger quantities of non-infective material intraspinally.

In these experiments, the details of which will be seen in Table 2, rabbits Nos. 1, 2, and 3 were injected intraspinally with material which had been kept in an ice box for 66 days and then in the light

at room temperature for 219 days. Rabbits Nos. 4, 5, and 6 were injected with material that had been kept in a north window, room temperature, for eleven months. When these six were injected the technic of making the spinal puncture was undeveloped and it was suspected at the time that the needle had not entered the spinal canal of rabbits Nos. 1 and 4. Their susceptibility to a later inoculation of 4 M.I.D. showed this suspicion to have been well founded. Rabbits Nos. 7 to 19 were injected with material which had been in the dark at room temperature for eight months. Rabbits Nos. 20 to 23 were injected with material which had been kept in the presence of P_2O_5, which, it has been demonstrated, causes a relatively rapid destruction of virulence.

TABLE 2.

Rabbit	Amount Injected	Time of Inoculation	Amount	Result
1	20 mg. f.v.	10 days later	4 M.I.D. f.v.	Died
2	20 " "	10 " "	4 " "	Immune
3	20 " "	10 " "	4 " "	"
4	20 " "	10 " "	4 " "	Died
5	20 " "	10 " "	4 " "	Immune
6	20 " "	10 " "	4 " "	"
7	25 " "	same time	1 " "	"
8	20 " "	" "	2 " "	"
9	5 " "	4 days later	5 " "	"
10	20 " "	4 " "	10 " "	Died
11	5 " "	6 " "	10 " "	"
12	10 " "	6 " "	15 " "	Immune
13	15 " "	6 " "	10 " "	"
14	5 " "	8 " "	20 " "	Died
15	10 " "	8 " "	20 " "	"
16	10 " "	8 " "	30 " "	"
17	15 " "	8 " "	20 " "	Immune
18	20 " "	14 " "	20 " "	Died
19	20 " "	15 " "	10 " "	Immune
20	20 " "	same time	2 " "	Died
21	20 " P_2O_5	14 days later	5 " "	Immune
22	20 " "	14 " "	10 " "	Died
23	20 " "	14 " "	20 " "	"
24	12 " s.v.	same time	4 " "	"
25	12 " "	14 days later	10 " "	"
26	20 " "	14 " "	20 " "	"
27	12 " "	28 " "	10 " "	Immune
28	20 " f.v.	11 " "	10 " "	"
29	25 " "	44 " "	10 " "	"

Rabbits Nos. 24 to 27 were injected with desiccated street virus whose infectivity had been destroyed by placing it in direct sunlight (west window) for three weeks. The virus was obtained from a case of rabies in a woman and the material was the brain of a rabbit, first passage. It had been desiccated five months. It will be seen that three of these rabbits were not immune to 10 M.I.D. 14 days

after the injection, but that the fourth resisted a similar inoculation 28 days later. The action of direct sunlight may have been a factor in lessening the immunizing capacity of the material. It is of interest to note that street virus may be used for immunization by this method.

Rabbit No. 28 was injected subdurally (trephine) with material which had been kept in an ice box for seven months and in one of the laboratory rooms in daylight for 39 days. Rabbit No. 29 is the same as No. 7 and after surviving this test was reinoculated 44 days later.

These experiments show that the brain of a rabbit may be directly immunized by a single intraspinal injection of non-infective material to such a degree that two weeks later the animal successfully withstands a subdural injection of 10 to 20 times an otherwise fatal dose. The immunity is acquired so rapidly that the intraspinal injection of five milligrams or more will protect against a simultaneous inoculation of a minimal infective dose injected subdurally (trephine).

Similar experiments were made on dogs as follows: Three dogs were injected by lumbar puncture with 50 mg. of non-infective material and 16 days later were inoculated under the dura with 1–10 mg. of the medulla of a rabid dog (desiccated).

In another series, one dog was given 10 mg. of a non-infective desiccated brain; two dogs 20 mg.; two dogs 40 mg.; and five were inoculated eight days later with 1–10 mg. of desiccated street virus. Two other dogs were injected with 50 mg. and 25 mg. intraspinally and at the same time were inoculated under the dura with 1–10 mg. of desiccated street virus. Two dogs were used as controls. The estimated amount of infective virus received by each of these twelve dogs was five times the infective dose for rabbits.

One of the control dogs developed furious rabies on the fourteenth day. The other control became emaciated five weeks later, refused food on the 44th day, and died on the 46th day after a paralysis which developed a few hours earlier. The autopsy did not reveal any cause of death and Negri bodies were not found in the brain. One of the two dogs inoculated and immunized at the

same time became extremely emaciated five weeks later, developed paralysis on the 36th day, and died on the 38th day. The remainder of the immunized dogs have shown no recognizable injury following the inoculation.

The above experiments seem to show that it is comparatively easy to immunize rabbits against rabies by a single intraspinal injection of non-infective desiccated "virus," and that the resulting immunity is acquired with surprising rapidity. The test on the dogs is somewhat unsatisfactory because the amount inoculated was too small to produce a definite and early outbreak of the disease in both control animals. It does appear, however, that the intraspinal injection is not followed by any ill results.

Whether or not fixed virus may be rendered non-infective by methods other than one I have employed without, at the same time, lessening its immunizing value, is a question which offers an opportunity for further investigation. It appears that the material whose infectivity was destroyed by P_2O_5 and by direct sunlight did not have so great an immunizing value as the material which had been allowed to die more slowly.

If further investigations show that the intraspinal injections of this material are harmless to animals, and that immunity is regularly and rapidly conferred, we may be in possession of a means of saving some of those patients who have received severe injuries about the face, and who now succumb in spite of the treatment employed at the present time. Another advantage of this method is that the immunizing property of the material I have used is not affected by age, and it may be stored in quantities and kept anywhere for immediate use.

NATURAL AND INDUCED IMMUNITY TO TYPHUS FEVER.*†‡

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Instances of transient or even permanent natural immunity of human beings to certain infections are not unknown. Most of these are clinical observations. It has been frequently noted that of a number of persons of a family or of a community presumably equally exposed, some escape. Those who have made many vaccinations for the prevention of smallpox, especially of children, are familiar with the fact that sometimes an individual may resist several repeated inoculations; this, while it may frequently be attributed to a non-potent virus, is in some instances, at least, undoubtedly due to a more or less transient natural immunity to vaccinia.

An interesting instance of natural immunity to an infectious disease in the human subject is reported by Reed.[1] One of the subjects experimented on by Reed and Carroll resisted a subcutaneous injection of 1.5 c.c. of yellow-fever blood and later resisted also the bites of some infected mosquitoes.

Similar examples of individual resistance of animals to experimental infection have been noted. In the course of our studies in measles we found quite a marked variation among monkeys in the susceptibility of different individuals to the disease. Marks[2] has well brought out the variation in susceptibility of young rabbits to poliomyelitis; and Dorset, McBryde, and Niles[3] report instances of natural immunity in the hog to hog cholera. Metchnikoff and

* Received for publication October 7, 1912.

† Read before Section 1 of the 15th International Congress on Hygiene and Demography, Washington, September 23, 1912.

‡ For protocols and fuller details see *Bulletin 86* of the Hygienic Laboratory, U.S. Public Health Service, on "Collected Studies on Typhus," paper No. 7, entitled "Studies in Immunity and Means of Transmission of Typhus," by John F. Anderson and Joseph Goldberger.

[1] *Med. Rec.*, 1901, 60, p. 201.

[2] *Jour. Exper. Med.*, 1911, 14, p. 116.

[3] Bur. Animal Industry, U.S. Dept. of Agric., *Bull. 102*.

Besredka[1] report that in one of 16 experiments they failed to infect the chimpanzee with typhoid; and McCoy and Chapin[2] report that a considerable percentage of San Francisco rats were found by them to be immune to plague at a time when immunity, due to a previous attack, could reasonably be excluded. Coming to typhus, we find that Nicolle[3] was unsuccessful in his initial attempts to infect monkeys directly from the human subject, and from his experience concluded that to obtain success the virus had to be prefaced by passage through a higher ape, the chimpanzee.

Our own work[4] and that of Ricketts and Wilder[5], of Gaviño and Girard,[6] and others, and more recently that of Nicolle himself, has shown that such prefatory treatment of the virus is unnecessary. The apparently ready susceptibility of the monkey encountered by us in our early work suggested to us that Nicolle's initial lack of success was due to the small amount of blood used, to the subcutaneous route employed by him in those inoculations, or to both combined.

Apart from these factors there appeared to us no reason to suspect natural resistance as playing a part in explaining Nicolle's negative results.

Gaviño and Girard appear to have been the first to suggest that some monkeys may be naturally immune to typhus. They reported that a monkey of the species *Ateles vellerosus*, that had previously received an injection of heated typhus blood, failed to present any reaction following an immunity test of 5 c.c. of virulent blood for the control. They suggest that, instead of the animal having been vaccinated by the first non-infecting injection, it may have been naturally immune to typhus.

Dreyer[7] reports that in the course of his work he found one monkey (*Cercopithecus?*) entirely refractory. This animal showed

[1] Metchnikoff, E., and Besredka, A., *Ann. de l'Inst. Past.*, 1911, 22, p. 192; *ibid.*, 1911, 25, p. 865.

[2] U.S. Public Health and Marine Hospital Service, *Bull.* 53.

[3] Nicolle, Ch., and Conseil, E., *Compt. rend. de l'Acad. des sci.*, 1910, 151, p. 598; *Ann. de l'Inst. Past.*, 1911, 25, p. 13; Nicolle, Ch., Conor, and Conseil, *ibid.*, p. 97; Nicolle, Ch., and Conseil, *Compt. rend. de l'Acad. des sci.*, 1911, 153, p. 1522; Nicolle, Ch., Conseil, and Conor, *Ann. de l'Inst. Past.*, 1912, 26, p. 250; Nicolle, Ch., and Conseil, *ibid.*, p. 275; *ibid.*, pp. 332, 334.

[4] *Jour. Med. Res.*, 1910, 22, p. 469.

[5] *Arch. of Int. Med.*, 1910, 5, p. 361.

[6] *Publicaciones del Inst. bact. nacional Mexico*, November 9, 1910.

[7] *Arch. f. Schiffs- u. Tropen-Hyg.*, 1911, 15, p. 319.

no trace of illness, although two others treated with the same material at the same time and in substantially the same dose promptly developed the usual (typhus) symptoms. He believed himself qualified in interpreting this as a case of natural immunity. He does not state whether he tested the susceptibility of this animal a second time.

Wilder[1] reports that a monkey that had received an injection of filtered typhus blood serum failed to react when given an immunity test of 4 c.c. of typhus virulent blood and suggests, among other possibilities, that the animal may have been naturally immune.

Nicolle and his co-workers in their interpretations do not appear to have considered the possibility of a natural immunity in the monkey. We believe, however, that a critical study of their reported protocols shows that they had encountered the same phenomenon.

In the course of our recent work with the New York and the Mexican virus we obtained results that brought the possibility of the occurrence of a natural resistance to typhus forcibly to our attention.

We have summarized all our monkey inoculations in which the first or primary inoculation was with monkey typhus blood. The virulence of the blood used and the dose, as well as the route of inoculation employed, unless otherwise stated, was always proven. It was found that when 46 monkeys were given a primary inoculation of virulent defibrinated blood, eight or 17.4 per cent, failed to react. These eight were given a second inoculation, to which four or 8.7 per cent of the original 46 failed to respond. It is proper to state, however, that one of these four may perhaps be considered to have presented a slight suggestion of an "abortive" reaction—so slight, however, that it escaped our attention at the time of its occurrence. If we count this as a positive reaction, we have only three of the eight that failed to respond to a second inoculation; or, in other words, at least 6.5 per cent of the monkeys failed to respond to two separate inoculations with virulent blood.

Of the three clearly resistant to the second inoculation, the immunity of only two was further tested; of these, one—rhesus

[1] *Jour. Infect. Dis.*, 1911, 9, p. 101.

No. 197—although given less than half its previous dose now developed a mild though well defined febrile reaction; the other, rhesus No. 189, presented no indication of a reaction. After this rhesus No. 189 was subjected to five more tests. In all, between January 2 and June 29 this animal received eight inoculations with virulent blood, ranging in amount from 3 c.c. to 14 c.c., to which no recognizable evidence of a reaction was noted at any time during the period of observation. Of 46 animals, therefore, one (2.2 per cent) failed to respond to any of the eight inoculations to which it was subjected.

We have summarized our experiments in which the initial inoculation was either unfiltered or filtered serum.

Five monkeys received a first inoculation of virulent unfiltered serum; three of the five animals failed to respond to this inoculation. Of these three, two responded promptly to the first immunity test (second virulent inoculation), while one, rhesus No. 221, has so far resisted five successive immunity tests (six virulent inoculations).

Seven monkeys received a primary inoculation of filtered serum; none of the seven responded to this initial inoculation. When subjected to an immunity test, however, all but two (Nos. 115*a* and 194) responded; in other words, two of the seven failed to react to the first injection of virulent material. One of these two (No. 194) responded to the second immunity test and the other (No. 115*a*) to the third.

If now we combine the results summarized in the foregoing, we find that of 58 animals, 13 or 22.5 per cent failed to react (i.e., did not become infected) after one injection of virulent blood or blood serum; five or 8.5 per cent failed to react after two injections; and two or 3.5 per cent after three injections. It is evident that a very large proportion (22.5 per cent) of monkeys possess at least a transient immunity and it seems reasonable to consider that in about 3.5 per cent of animals the resistance noted amounts to a permanent immunity.

It may perhaps be objected that this is only an apparent immunity, that the resistance is simply due to a virus of low or varying virulence, to the smallness of the dose employed, the site of inoculation, or to the size or age of the animal.

It is readily conceivable that different strains of virus may differ markedly in virulence. Analogies readily suggest themselves, and the results of our inoculations with virus from human sources appear to furnish some experimental support for such a view. A summary of our inoculations with blood from human cases of typhus shows that only a small proportion of cases, whether New York or Mexican, were successful.

While it is not improbable that in these cases viruses of differing virulence may have entered as an element, we do not believe that this factor enters into the results of the series of inoculations above considered, for the inoculations in question were made with a single strain which we have successfully propagated through some 23 monkey generations, throughout which the virus, so far as we are able to discern, has maintained its original degree of virulence. The suggestion that age may enter as a factor in susceptibility has been advanced because it is held by some that children are less susceptible than adults. As a matter of fact, there is no good evidence to show that children are less susceptible to typhus than adults. It is probably true that the manifestations of typhus in a child are not in all respects like those in the adult; that is, they are not typical, but this manifestly cannot be regarded as indicating a difference in the degree of susceptibility. That this and the other objections cited are not valid may be clearly inferred from the fact, first, that of two monkeys of substantially the same size and vigor, both inoculated at the same time, by the same route but with different quantities of the same virus, the animal receiving the larger dose (by 50 per cent) has failed to react; and, second, as has already been noted above, an animal may react after the second or the third inoculation, although the dose may be only half as large as that given in the immediately preceding ineffective inoculation.

The existence of a more or less marked transient (or permanent) natural immunity is of great practical importance in all work on typhus. Great caution and conservatism must be observed in the interpretation of experimental results, especially negative results. We believe, too, that such immunity may have a much broader significance and application. It will be recalled that Metchnikoff and Besredka, in their studies on antityphoid vaccination conclude

that the ingestion of the bacillus paratyphoid-B may vaccinate against true typhoid. This conclusion is based on the observation that in one of two experiments, a chimpanzee that had previously been infected experimentally with paratyphoid-B was subsequently refractory to infection with typhoid. Now, while the results of future work may reinforce this conclusion the thought readily suggests itself that an occasional chimpanzee may be met with that may be transiently or permanently resistant to infection with typhoid. Indeed, these authors themselves, in a previous communication report that they met with only one failure in 16 experiments to infect anthropoids. We believe, therefore, that their conclusion is to some extent at least, if not altogether, invalidated.

In a discussion of the susceptibility of the monkey it is essential to have a clear understanding of what one is to consider as a tyhpus reaction in this animal. Following an inoculation with virulent material the monkey continues to remain normal for a period varying from five to 24 days. As may be seen from Table 1 in about 90 per cent of cases the incubation period varies between six and 10 days.

TABLE 1.

Incubation Period	Number of Monkeys
5 days	1
6	12
7	20
8	16
9	28
10	16
11	4
14	4
15	1
24	1
Total	103

At the end of this period the temperature of the susceptible animal rises fairly rapidly as a rule, sometimes gradually or at times very abruptly. The fever reaches its fastigium in 36 to 48 or 72 hours; it then continues for a variable period of one or two to five or more days, then defervesces. The defervescence, like the invasion, is variable. Although usually gradual, it is frequently rapid or even critical. In brief, the course of the fever in the monkey is essentially like that of the fever in man.

The fever may be accompanied by loss of appetite, thirst, a ruffling of the fur, and a drooping posture; very commonly, however, even with a well defined febrile reaction, the animal, except for some slight listlessness, shows hardly any outward manifestations. In other words, the fever is the only definite index of a reaction. As may be seen from Table 2, in about 76 per cent of the cases the fever varies in duration between six and 10 days.

TABLE 2.

Duration of Illness	Number of Monkeys
3 days	2
4	1
5	1
6	5
7	9
8	24
9	21
10	17
11	9
12	6
13	3
14	2
15	1
29	1
Total	102

At the termination of the fever there is almost always manifest some degree of emaciation. Occasionally after the temperature has been normal for three or four days or a week it may go up a second time. Such a relapse may last five or seven or more days, and may end in recovery or death. We have met with febrile recrudescense or relapse in four of 105 cases.

Although in appearance a mild disease, we have thus far had four deaths in a total of 105 cases of typhus in the monkey. This total of cases includes 10 induced with the Mexican virus. Segregating these, we have four deaths in 93 cases of the disease induced with the New York strain (Brill's disease), a mortality notably higher than that reported by Brill in the human subject.

An animal that has presented a reaction such as above described is immune from subsequent infection. In Table 3 we present the results of immunity tests in monkeys which had presented typical primary reactions. It will be seen that in no instance has such an animal responded to a subsequent immunity test. Such an immu-

nity may last a long time, as is shown by the following: "Adela," a female rhesus, was originally infected by an intraperitoneal injection of 6 c.c. of defibrinated blood from a patient with (Mexican) typhus, January 11, 1910. This animal developed a marked typhus terminating January 29–30, 1910. Between November 9, 1911, and March 6, 1912, this monkey was given four inoculations of typhus blood, all of which it resisted absolutely, showing that it was still immune after two years.

TABLE 3.

RESULTS OF IMMUNITY TESTS IN ANIMALS THAT HAD PREVIOUSLY PRESENTED A MARKED REACTION.

Rhesus Monkey No.	Date	Cubic Centimeters Inoculated	Source	Site	Result
95..........	Nov. 23, 1911	3	Rhesus 306	Vein	—
	Dec. 8	3.5	Case 19M*	Peritoneum	—
127..........	Dec. 30, 1911	6	Case 38M	"	—
	Jan. 1, 1912	4	Case 39M	"	—
133..........	Nov. 23, 1911	3	Rhesus 306	Vein	—
	Dec. 8	3.5	Case 19M	Peritoneum	—
142..........	Nov. 29, 1911	5	Rhesus 170	Vein	—
	Dec. 12	4	Rhesus 161	"	—
	Jan. 11, 1912	7	Rhesus 186	Peritoneum	—
158..........	Nov. 9, 1911	5	Case 1M	Vein and subcutaneous	—
	Dec. 2	2.5	Case 16M	Vein	—
	Dec. 22	4.5	Case 26M	Peritoneum	—
188..........	Mar. 30, 1912	3	Rhesus 234	"	—
210..........	"	3	"	"	—
306..........	Dec. 29, 1911	6	Case 35M	"	—
	Jan. 1, 1912	4	Case 39M	"	—
	Feb. 1	2.5	Rhesus 184	Vein	—
315..........	Feb. 1, 1912	2.5	"	Vein	—
316..........	"	2.5	"	Vein	—
	Feb. 23	6	Rhesus 200, 203, 204	Peritoneum	—
317..........	Jan. 10, 1912	3	Rhesus 187	Vein	—
318..........	"	3	"	Vein	—
500..........	Nov. 9, 1911	4	Case 1M	Vein	—
	Dec. 2	2.5	Case 16M	Vein	—
	Jan. 10, 1912	3	Rhesus 187	Vein	—
	Mar. 6	6	Rhesus 213, 312	Peritoneum	—

* M = Mexican case.

Ordinarily, therefore, a well marked febrile reaction may be interpreted as typhus without subjecting the animal to an immunity test. When, however, the fever is slight or its course atypical, that is, when we have what may be designated as an "abortive" fever, this cannot be construed as a typhus reaction unless the immunity test proves the animal to be resistant to infection and even then not without some reserve. Should the immunity test in such cases show that resistance has not been conferred, a diagnosis of previous typhus is not permissible.

CONCLUSIONS.

1. Instances of a transient or permanent natural immunity of the monkey to typhus are not uncommon. In our experience 22.5 per cent of monkeys have failed to react to a first and 3.5 per cent to three or more successive inoculations with virulent blood or blood serum.

2. Repeated inoculations of virulent blood or blood serum, when not followed by a febrile reaction, confer no appreciable resistance. Failure of an animal to react to an immunity test cannot, in the absence of a previous febrile reaction, be interpreted as indicating that it was protected (vaccinated) by the primary, apparently ineffective, inoculation.

3. A typical febrile reaction has, in our experience, always conferred complete protection to subsequent infection; in one instance for at least two years. Such a fever, therefore, under experimental conditions, justifies a diagnosis of typhus.

4. An atypical or poorly defined ("abortive") fever following inoculation cannot be interpreted as typhus unless it is followed by resistance to subsequent immunity tests.

IMMUNIZATION BY MEANS OF CULTURES OF TRYPANOSOMA LEWISI.* †

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In a previous paper[1] it was shown that cultures of *Tr. lewisi* when plasmolyzed in collodium sacs, against distilled water, underwent changes resulting apparently in complete solution or rather disintegration of the organisms. Three or more injections, on alternate days, of such plasmolyzed cultures protected young rats against a minimal infective dose of virulent blood. Immunity could therefore be produced by the injection of disintegrated cultural trypanosomes.

At the time it was also pointed out that cultures of *Tr. lewisi* and *Tr. brucei*, even after they had passed through a hundred generations or subcultures, in the course of two years, do not become attenuated by such prolonged consecutive passage but readily infect. This statement is only partially true, since the organisms on prolonged cultivation actually do suffer some loss of virulence or capacity to infect. A culture of *Tr. brucei* in the 100th generation will infect rats but the period of incubation is somewhat lengthened, and while death may occur within two or three weeks, it may not result until several months have elapsed from the time of inoculation; the longest survival is one which is at present in its 253d day. Obviously, the conditions which obtain in cultivation, notably the composition and reaction of the medium, the temperature and duration or age of culture, are not without appreciable effect on these organisms.

From the beginning, when the cultivation of *Tr. lewisi* and *Tr. brucei* was first effected, we have looked forward to the production of attenuated strains which could be utilized in preventive inoculations. It seemed reasonable to believe that the cultures of

* Received for publication August 24, 1912.

† This research has been aided by a grant from the Rockefeller Institute for Medical Research.

[1] *Proc. Soc. Exp. Biol. and Med.*, 1907, 4, p. 42.

pathogenic protozoa, like those of bacteria, would be found available to some extent in the production of immunity. The fact that spontaneous recovery may occur in protozoal diseases, as is the case with relatively insusceptible species, indicates the establishment of an acquired immunity, and hence the inherent capacity possessed by such an animal to protect itself against the given parasite. The trypanosomal infection of the common rat is a most excellent illustration of this fact, since it only exceptionally proves fatal. In the course of some weeks, or months, the rat gets rid of the trypanosome and becomes immune to a subsequent inoculation. It is evident, therefore, that the cultures of this parasite are particularly suitable for work bearing upon trypanosomal attenuation and immunity. With this organism it has been possible to demonstrate two important facts, first, that attenuation can be secured, and second, that such living, attenuated cultures confer a solid and permanent immunity.

The culture of *Tr. lewisi* which we have used was isolated from a rat September 5, 1905, since which time it has been maintained on ordinary blood agar medium, at 25°. It is at present in the 313th generation. At first, the culture was readily infective; the growth from a single tube being sufficient to produce typical infection in the rat. This infective property persisted for a considerable period and was frequently utilized to supply infected rats for the laboratory. After a long interval, in March, 1909, this procedure to secure a rat infection was again resorted to, the culture then being in the 158th generation. Contrary to expectation, no infection resulted. During the next few weeks no less than 17 rats were tested by intraperitoneal injections, each receiving from two to four cultures. In every case, as before, the result was negative, although daily examinations were made for an entire month. These tests clearly demonstrated that this stock culture of *Tr. lewisi* had completely lost its capacity to develop within the rat. In other words, it had become fully attenuated.

The many inoculations made with this culture during the past three years have invariably failed to produce infection. The result is the same whether the contents of a single tube or of 50 tubes is injected into the rat. Such cultures, grown at 25° for seven to 10

days, were extremely rich in flagellates which at times formed a thick slimy covering on the blood agar medium. Hence, the quantity of flagellates injected was at times enormous, and yet, notwithstanding this fact, no infection could be produced. The microscopic appearance of the cultures was in no wise different from that of the early generations which gave positive results, but the parasitic property of the organism was definitely lost.

All attempts to restore the infectious character to the culture have been fruitless. Even when the culture was placed in collodium sacs, in the peritoneal cavity of rats, it failed to multiply. Possibly further efforts in this direction may be successful.

While it is true, as has been emphasized above, that the cultures are no longer infective, it is an interesting fact that the majority of the inoculated rats do show in their blood, within the next day or two, a small number of fairly typical blood trypanosomes. These, however, do not increase in numbers and usually disappear within 48 hours after the injection. After that they are not to be found even though most careful examinations are made for a month or more. With the hope of restoring the virulence, the blood of rats showing these early trypanosomes was drawn from the heart and injected[1] into clean young rats and was also planted at the same time on blood agar. Much to our surprise, it was found that such blood failed to infect the rats and also failed to give cultures in the tubes.

The failure to secure a growth on the blood agar medium was particularly surprising, since, as is well known, this cultural method is capable of revealing trypanosomes even when prolonged microscopic examinations are negative. In all, about 12 rats which showed these transitory trypanosomes were cultured without obtaining the slightest indication of a growth. Similarly, the inoculation of nine rats with such blood, the doses varying from 0.25 c.c. to 3 c.c., failed to infect. Here, as above, the negative result could not be ascribed to the small number of trypanosomes, for it can be readily shown that the normal trypanosomal blood will infect, when diluted, to such an extent that the parasites can scarcely be detected under the microscope. Likewise, the suscep-

[1] All injections unless otherwise indicated were given intraperitoneally.

tibility of our rats could not be called into question. In all of our work special care was taken to use only young rats, weighing from 60 to 90 gms., which had been repeatedly examined for two or more weeks and thus found to be free from natural infection. When used as controls, the injection of 1 c.c. of a 1:10,000 dilution of blood containing *Tr. lewisi* invariably gave a rich infection.

It is evident, therefore, that the trypanosomes which appear transitorily in the blood of rats after the injection of a culture are incapable of further multiplication or reproduction. They are sterile or abortive forms, and without doubt their further study will prove of interest. It would seem as if some of the cultural trypanosomes still possessed a tendency to develop into the blood form, but the resulting individual through lack of inherent vigor or failure of fertilization (?) is unable to develop further and soon disappears.

It is probable that these abortive forms are present in the blood of all rats inoculated with the cultures. They are, however, easily overlooked and for that reason they have been found in only 75 per cent of the rats examined. They have been observed in the blood on one occasion five hours after the injection; at other times within 12 hours, but more often between 24 and 48 hours. Only exceptionally have they been seen as late as 72 hours and once they were observed to be present for four days. As already stated, the abortive forms are to be found in small numbers only. At times, after a prolonged search, but a single one can be found; more often, one can be found for every five or ten fields (No. 7 objective); and, rarely, one for every two fields examined. The number of abortive forms is independent of the dose injected, for in rats which received 50 cultures they were no more numerous than when a single culture was given. Likewise, the age of the culture seems to have no influence upon the number of these forms.

IMMUNITY.

As already stated, our previous studies have shown that cultures of *Tr. lewisi* when plasmolyzed[1] were capable of producing immunity

[1] This method of preparing a vaccine, though not generally applicable, has been utilized in this laboratory by Dr. J. G. Cumming, in charge of the University Pasteur Institute, in the preparation of a safe antirabic vaccine. His experiments have shown that the dialyzed virus protects animals even more efficiently than does the desiccated virus prepared in the ordinary way.

in rats. It was therefore to be expected that the living attenuated cultures would behave in a similar way and confer protection against the blood trypanosome. The experimental work in this direction demonstrated that such was the case and that an active immunity was developed in animals which had received single or multiple injections of the living cultures.

In order to obtain absolutely conclusive results it was necessary to employ young rats, preferably those raised in the laboratory, which repeated examinations for at least two weeks before use had shown to be free from the natural infection. In the beginning, when rats which had been purchased on the market were used, it not infrequently happened that the control animals in an experiment would fail to become infected, thus invalidating the immunity test. This difficulty was entirely overcome by employing only carefully tested young rats.

Another point of importance, not infrequently lost sight of in immunity tests, is the use of a very small but surely infective dose of the virulent organism, for the reason that a given degree of immunity may be effective against a minimal infecting dose but not against a hundred or thousand doses. A very large dose of virus may readily break down a slight immunity and thus lead to the erroneous conclusion that no protection exists. With this consideration in mind, we have as a rule employed the test virus in a dilution (in 0.85 per cent salt solution) of 1:10,000. One cubic centimeter of this suspension has never failed to produce a rich infection in clean young rats. In some instances, as shown in the tables, suspensions of 1:200 or 1:2,000 were used.

On examination of Table 1, several interesting facts will be noted. When the interval between the injection of the vaccine and the test dose is but two or three days (rats Nos. 3, 20, 21, 9, 27) a rich infection results, showing that little or no immunity exists at that time. Possibly a smaller dose of the test virus might have shown some immunity at this early date, because this is certainly evident on the fourth day (Nos. 5 and 23). On the other hand, but one of the two rats inoculated on the eighth day (No. 28 which received six cultures) developed sufficient resistance to abort the infection.

TABLE 1.

Immunity Tests Made after a Single Injection of

2 Cultures

Rat No.	Interval between Injections	Test Dose of *Tr. lewisi* Blood	Result	Remarks
1	14	0.005 c.c.	⊕	1 trypanosome on third day
2	14	" "	o	
3	2	0.0005 "	+	Rich infection
4	18	" "	o	
5	4	0.0001 "	o	
6	9	" "	o	
7	13	" "	o	
8	16	" "	⊕	Very mild infection; 1 per field for seven days
9	3	" "	+	Rich infection
10	8	" "	+	" "
11	12	" "	o	
12	16	" "	o	
13	10	" "	o	
14	10	" "	⊕	Very mild infection, 1 per field for nine days
15	10	" "	o	
16	10	" "	o	
17	10	" "	o	

6 Cultures

Rat No.	Interval between Injections	Test Dose of *Tr. lewisi* Blood	Result	Remarks
18	14	0.005 c.c.	o	
19	14	" "	o	
20	2	" "	+	Rich infection
21	2	0.0005 "	+	" "
22	18	" "	o	
23	4	0.0001 "	⊕	Very mild infection; 1/10 per field for three days
24	9	" "	o	
25	13	" "	o	
26	16	" "	o	
27	3	" "	+	Rich infection
28	8	" "	⊕	Very mild infection; 1 per field for six days
29	17	" "	o	
30	17	" "	o	
31	17	" "	o	
32	17	" "	o	
33	17	" "	⊕	Very mild infection; 1–5 per field for 4 days

In the tests tabulated above, controls were inoculated each time after the experimental animals, and developed a rich and lasting infection.

o indicates no infection, i.e., immunity is present.

+ indicates a rich infection, the same as that in the controls.

⊕ indicates a mild or abortive infection characterized by the presence of very few trypanosomes per field in fresh blood preparation.

Very mild or abortive infections may occur, as will be seen, in rats inoculated even on the 10th (No. 14), 14th (No. 1), and 17th (No. 33) day, but in these the parasites were, as a rule, very scanty and persisted for but a few days. These infections correspond to those which can be produced by injecting a large dose of trypanosomal blood into rats which have recovered from a natural infection. The breaking down of the immunity in such cases is usually indicated by a mild infection of but a few days' duration.

Altogether it will be evident that immunity is produced by the injection of living cultures of *Tr. lewisi* and that this immunity is most pronounced after the lapse of about 10 days. In the experiments discussed, the smallest dose of vaccine was represented by the growth present in two culture tubes.[1] The minimum dose of the living culture, sufficient to immunize, is probably a very small fraction of that mentioned. Thus, in one experiment, four rats were inoculated with the growth corresponding to one-fourth, one-half, one, and two culture tubes, respectively. After an interval of 12 days, these rats and two controls were inoculated with 0.0001 c.c. of trypanosomal blood. The treated rats showed no infection, whereas the controls became heavily infected.

Subcutaneous injection of cultures.—As might be expected a notable difference in immunity production is obtained according to whether the vaccine is introduced intraperitoneally or subcutaneously. When introduced by the latter route a much larger dose of the culture must be given in order to obtain protection. Only one experiment with single injections, given subcutaneously, was made and is worthy of note. As shown in Table 2, the rats which received two cultures became infected as heavily as did the controls; whereas those which received six cultures at the same time showed a mild abortive infection which persisted for one and three days, respectively. Evidently a single injection given subcutaneously is much less effective than when given intraperitoneally, for while a fraction of a culture is sufficient when administered by

[1] The tubes used for cultures were small, being but 12×150 mm. After inoculation the tubes were placed in a hot room at 25° for from seven to 12 days. The growth was then taken up in 0.85 per cent salt solution and at once injected.

It may be stated in this connection that the injection of an extract from uninoculated tubes has no immunizing action.

the latter route, a much larger dose is necessary when injected under the skin.

When several injections are given subcutaneously, the resulting immunity is much more marked, since the absorption of the antigen is necessarily greater. One experiment of this kind is given in Table 2 and shows perfect protection.

TABLE 2.

IMMUNITY TESTS AFTER SUBCUTANEOUS INJECTION OF LIVING CULTURES.

Rat No.	Received	Interval before Test Dose	Test Dose of *Tr. lewisi* Blood	Result	Remarks
1	2 cultures	18 days	0.0001 c.c.	+	Rich infection
2	" "	" "	" "	+	" "
3	6 cultures	" "	" "	⊕	Mild infection lasting 3 days
4	" "	" "	" "	⊕	" " " 1 day
					Two controls gave a rich infection
5	5 injections in 5 days each of 2 cultures	17 days	" "	o	Perfect immunity
6	5 injections in 5 days each of 2 cultures	" "	" "	o	" "
7	5 injections in 5 days each of 2 cultures	" "	" "	o	" "
					Four controls gave a rich infection

Multiple injections.—In Table 2, Nos. 5, 6, and 7 show that multiple injections of the culture given subcutaneously impart a marked degree of immunity. A number of similar tests were made, the injections being intraperitoneal, in order to obtain hyperimmunity and to test the resistance of such rats to relatively large doses of trypanosomal blood. The rats thus immunized, while fully protected against a minimal dose of virus, on the injection of a fairly large dose, such as 0.25 c.c., may show a mild abortive infection which disappears in from two to three days. An exactly similar "break down" of immunity frequently occurs when a large dose of infective blood is injected into rats which have spontaneously recovered from an infection with *Tr. lewisi*. These abortive infections depend largely upon the dose and to some extent upon the rat itself.

The result of these tests is given in Table 3. By way of explanation it should be stated that Nos. 1–4 received in the course

of 12 days three injections each of six cultures, Nos. 5–7, in 58 days, received three injections, each of two cultures, while Nos. 8–10 in the same time were also given three doses, each of four cultures and Nos. 11–13 received similar injections, each of six cultures. Nos. 14–21 were given in the course of 67 days six injections, the first four receiving two cultures each time and the last four, six cultures.

TABLE 3.

IMMUNITY AFTER MULTIPLE INJECTIONS.

Rat No.	Received	Interval before Test Dose	Test Dose of *Tr. lewisi* Blood	Result	Remarks
1.......	3 × 6 C 12 days	13 days	0.25 c.c.	⊕	Tr. present for two days
2.......	" " " "	" "	" "	⊕	" " " three "
3.......	" " " "	" "	" "	o	Full protection
4.......	" " " "	" "	" "	⊕	Tr. present for two days
					Four controls gave rich infection
5.......	3 × 2 C 58 days	5 days	0.1 c.c.	o	
6.......	" " " "	" "	" "	o	
7.......	" " " "	" "	" "	⊕	Tr. present for four days
8.......	3 × 4 C " "	" "	" "	o	
9.......	" " " "	" "	" "	o	
10.......	" " " "	" "	" "	o	
11.......	3 × 6 C " "	" "	" "	o	
12.......	" " " "	" "	" "	o	
13.......	" " " "	" "	" "	o	
					Two controls for this set gave rich infection
14.......	6 × 2 C 67 days	7 days	0.005 c.c.	o	
15.......	" " " "	" "	" "	o	
16.......	" " " "	" "	" "	o	This set of eight were injected two weeks later with 0.25 c.c of virulent blood. All resisted perfectly this secon injection
17.......	" " " "	" "	" "	o	
18.......	6 × 6 C " "	" "	" "	o	
19.......	" " " "	" "	" "	o	
20.......	" " " "	" "	" "	o	
21.......	" " " "	" "	" "	o	
					Four controls for this set gave rich infection

DURATION OF IMMUNITY.

Since a single injection of the living culture protects against a surely infective dose of *Tr. lewisi*, it was of interest to ascertain the duration of such immunity. This active immunity becomes manifest in about 10 days and once developed it probably persists until the death of the animal. Up to the present time we have not tested out the immunity for a longer period than five months, but there can be little doubt that it will be found to last for a considerably longer period.

In Table 4, which may be looked upon as a continuation of Table 1, are brought together the several tests in which the interval

between the injections exceeds 18 days. The several groups were tested at different times with the usual dose of 0.0001 c.c. of trypanosomal blood. In every case controls were inoculated at the same time and invariably gave a rich infection.

TABLE 4.

Duration of Immunity Following the Single Injection of Living Cultures.

Rat No.	Received	Interval before Test Dose	Test Dose of *Tr. lewisi* Blood	Result	Remarks
1	2 cultures	35 days	0.0001 c.c.	o	
2	" "	" "	" "	o	
3	6 "	42 "	" "	o	
4	" "	" "	" "	o	
5	6 "	74 "	" "	o	
6	" "	" "	" "	o	
7	2 "	78 "	" "	o	
8	" "	" "	" "	⊕	A single trypanosome was found on each of two days
9	" "	84 "	" "	o	
10	" "	" "	" "	o	
11	" "	112 "	" "	o	
12	6 "	119 "	" "	o	
13	2 "	150 "	" "	o	
14	6 "	159 "	" "	o	

IMMUNIZATION AGAINST NATURAL INFECTION.

The marked protection following the introduction of living cultures suggested the possibility that vaccinated rats would be equally protected against natural infection by the flea or louse. The number of parasites present in the test dose of blood employed in the previous experiments must be considerably larger than can be introduced through the agency of a biting insect. If, therefore, no other factor is involved, it should be possible to protect the rats against the natural infection by means of insects.

The white rat which we have used exclusively has never been found in this laboratory to harbor fleas and consequently we have been unable to undertake experiments with this insect. The rats, however, are often heavily infected with lice, and since, unquestionably, these as well as fleas are agents of transmission, we endeavored to protect the rats against this source of infection.

In our first experiment we tried to secure infection by the direct transfer of lice from infected rats to a set consisting of four clean and four vaccinated rats, each of the latter having previously been

given an injection of six cultures. Although considerably over 100 lice were transferred to each rat, no infection followed. Later on, therefore, these rats were confined in a small space with infected rats which were heavily infested with lice but the result was no better. At the end of five months, although blood examinations were made twice weekly for over four months, no trypanosomes could be detected in the exposed rats. The failure of the controls to become infected was not due to an unrecognized infection, since one of these inoculated on the 133d day with 0.0001 c.c. of virulent blood gave a typical infection.

For the second experiment 10 rats were injected each with six cultures. Five of these (see Table 1, Nos. 29–33) were tested 17 days later and found to be immune. The remaining five, together with five clean rats for control, and five rats containing *Tr. lewisi* and heavily infested with lice, were placed in a small cage where they remained for several months. The rats were examined at least once a week for two months but no spread of the infection occurred.

A third experiment was more successful. As above, 10 rats were first injected each with two cultures and after 10 days, five of these were tested and found to be immune (see Table 1, Nos. 13–17). The remainder, together with five clean and eight lousy, infected rats, were confined in a small cage. Later five additional infected and lousy rats were added. Of the vaccinated rats four died on the 24th, 29th, 52d, and 130th day, respectively, without at any time showing trypanosomes in their blood. Of the five clean controls, two died on the 61st and 106th day without becoming infected. The remaining three became infected on the 28th, 69th, and 81st day. The fact that three out of five non-immunes and none of the vaccinated animals became infected would indicate that the living vaccine does protect against infection by the louse. Further tests, however, especially with directly transferred lice, will be necessary fully to establish this point. It seems from the work done thus far that the lice from young rats which have been inoculated with *Tr. lewisi* but a few days before are more likely to convey the infection than those from rats having an old chronic infection.

CURATIVE ACTION OF CULTURES.

The readiness with which the living culture immunizes against infection opens the possibility of using such material to abort, or in other words, cure an infection already under way. The extremely rapid and heavy infection in the case of *Tr. lewisi* makes this at the outset a very unpromising attempt; for when a relatively small dose of trypanosomal blood, as shown in Table 3, may serve to break down an existing immunity it is very probable that the enormous number of parasites in the infected animal will exert a corresponding action and hence completely overwhelm any resistance which might be produced by the vaccine. Possibly repeated inoculation of large quantities of vaccine might eventually give rise to an amount of antibodies sufficient to check the parasites present.

Before, however, undertaking to put an end to a well developed infection, it was desirable to ascertain whether it was possible to check an infection at the very beginning; in other words, to prevent it by the simultaneous injection of virus and vaccine. In one experiment of this kind, three young rats received an intraperitoneal injection of 0.0001 c.c. of trypanosomal blood. Two of these were reserved for controls while the third was given, likewise intraperitoneally, within an hour, a suspension of the growth from 50 cultures. A fourth rat also received 50 cultures and served as control. It showed a few abortive trypanosomes on the first two days but otherwise no sign of infection. Trypanosomes did not appear in the blood controls until the third day, and then in very small numbers, about one in every 10 fields of the fresh blood. In the treated rat, on the contrary, they appeared on the first day, were two per field on the second, and four per field on the third day. Some blood drawn from the tail of this rat on the second day and injected into another produced a typical infection, thus demonstrating that the trypanosomes present were not of the abortive type. The appearance of the trypanosomes in the treated rat so much earlier than in the untreated controls is an interesting fact which was corroborated in the second experiment.

Although the trypanosomes in the untreated controls appeared later than in the treated rat, yet, once present, they multiplied more rapidly and in three days were several times more numerous

than in the latter. On the seventh day they approximated, roughly speaking, about 200 per field, and this rich infection was maintained during the following three weeks. In the treated rat the maximum number was about 100 per field, on the eighth to the 11th day; the parasites then decreased and disappeared on the 30th day, at which time both controls still showed a maximum infection. This experiment indicated that the injection of a sufficiently large dose of vaccine, shortly after the infective dose, modifies somewhat the course of the infection. The result, however, was not as sharp and conclusive as might be desired.

Consequently, a second experiment was made. Two rats were given an infective dose of 0.0001 c.c., as before. One of these was given 100 cultures in three injections, during the next three hours. In this treated rat, as in the one mentioned above, trypanosomes were found on the first and second days, at which time none could be found in the control. Stained preparations made at this time showed that the trypanosomes were nearly all of the large "female" type, many of which were undergoing active division as indicated by the presence of double nuclei, blepharoplasts, and even of double flagella. Minute flagellates, such as are formed by the breaking up of the eight-celled rosette, were also noted. Of special interest is the fact that though the trypanosomes appeared earlier than in the control they did not continue to increase as they did in the latter.

In the treated rat, the trypanosomes at first appeared to be stimulated to very rapid multiplication, but this was soon checked and the number was greatly decreased, so that on the sixth and seventh days, on an average, not more than one could be found in every two fields of a fresh blood preparation. After this there was a gradual rise and although the rat was examined for 26 days the number never exceeded about 75 per field. The course of the infection in this treated rat, even more so than that in the first experiment, is indicative of a marked inhibitory influence and points to the possibility of aborting an infection by repeated large doses of the vaccine.

The course of the infection in the rats of this last experiment can best be seen from the following summary. The figures here given express roughly the number of trypanosomes present, per field of

the No. 7 objective, of a fresh blood preparation. Thus, 1/10 is to read as one trypanosome in 10 fields; 3 as that many per field, etc.

Inoculated	Examined											
Feb. 26	Feb. 27	Feb. 28	Feb. 29	Mar. 1	Mar. 2	Mar. 3	Mar. 4	Mar. 5	Mar. 6	Mar. 7	Mar. 8	Mar. 9
Treated rat...	1/10	3	10	10	5	½	½	5	10	10	25	50
Control rat...	0	0	1/20	15	75	100	150	200	200	200	250	Dead

PASSIVE IMMUNITY.

Since the animals treated with one or more injections of living cultures develop a very marked immunity, it was to be expected that their blood would carry antibodies, and consequently no special effort has as yet been made otherwise to demonstrate their presence. Three tests, however, of a preliminary character, were made with the blood of rats which had received several spaced injections. These tests, like the curative experiments given above, were intended to ascertain the effect of simultaneous injections of virus and hyperimmune blood. The results, it will be seen, are by no means satisfactory.

Experiment 1.—The immunized rat received, in the course of 28 days, seven injections, each of four cultures. After an interval of 153 days, 1 c.c. of blood was drawn from the heart and injected into a clean young rat. This was followed at once by an injection of 0.0001 c.c. of trypanosomal blood. A like dose of the virus was given at the same time to two controls and to the hyperimmune rat. The controls developed the typical rich infection, whereas the latter, as expected, resisted perfectly, although five months had passed since the last protective inoculation. The rat which received the dose of immune blood became infected the same as the controls. Apparently, the blood was without protective action.

Experiment 2.—The immunized rat received four injections, of six cultures each. Seventeen days after the last inoculation it was bled and two rats were injected with the blood, receiving 2.0 and 0.5 c.c., respectively. Within a minute, these two rats and two controls were each given 0.0001 c.c. of trypanosomal blood. The rat which received 2 c.c. of the immune blood had a period of incubation of five days; the other three rats showed parasites on the third day. Otherwise, there was no difference, the severity of the infection in the treated rats being the same as in the controls. Here again, the blood seemed to be without any protective action.

Experiment 3.—The immune rat in this test had been given a total of 34 cultures in six injections. It was bled after an interval of 31 days and two rats were given respectively, 3.0 and 1.0 c.c. of its blood. This injection was followed within four minutes by the usual dose of trypanosomal blood (0.0001 c.c.). The same dose of

virus was given to two controls and to two young rats born of a mother which had received an injection of two cultures. The latter and the controls gave rich infections. The treated rats, while they possibly showed fewer trypanosomes on the first two days of the infection, eventually gave as rich infections as the controls.

It is evident that in these three tests passive immunity was not conferred. Had the immune blood been injected one or two days previous to the infective dose, the result might have been different. To protect against the virus, when given simultaneously, a much larger dose of blood is needed, or else the blood must come from animals which are more highly immunized.

EFFECT OF BERKEFELD FILTRATES OF PLASMOLYZED CULTURES.

Since living as well as dead plasmolyzed cultures immunize against infection with *Tr. lewisi*, it may be pertinent to inquire whether this immunity is due entirely to the solid intracellular constituents or whether soluble products are in any way concerned. In order to obtain some light on this point, a large number of cultures (120) were prepared. The growth was taken up in 0.85 per cent salt solution and transferred to collodium sacs, immersed in running distilled water, and subjected to plasmolysis for 18 hours. At the end of that time the flagellates had disintegrated, or rather shrunk to small rounded forms. The contents of the sacs were then centrifugated, at 8,000 revolutions, to remove the cell detritus, and the clear liquid was passed through a small Berkefeld filter. The filtration, aided by an aspirator, was rapid and required but a few minutes.

The clear filtrate was injected into 6 pairs of rats; the rats of each pair receiving the equivalent of 2, 4, 6, 12, 12, and 24 cultures, respectively. Twelve days later, each of these, as well as four controls, received 0.0001 c.c. of trypanosomal blood. One of the treated rats which received the equivalent of four cultures gave a mild infection which cleared up on the ninth day. All of the other treated rats developed as rich infections as did the controls and, consequently, the single exception referred to is of no significance. The only conclusion, therefore, which can be drawn from this test is that the Berkefeld filtrates of plasmolyzed cultures have no immunizing properties. It may be added, parenthetically, that the

same fact has been noted by Dr. Cumming in connection with his studies (unpublished) on rabic virus. While plasmolyzed suspensions of this virus confer a marked immunity, the filtrates of such suspensions are inert. In other words, it would seem that the insoluble constituents of the cultures, as well as of the rabic virus, are essential to the production of the immunity.

SUMMARY.

Tr. lewisi after cultivation for several years on rabbit blood agar medium becomes non-infective.

Trypanosomes may appear in very small numbers in the blood of rats inoculated with such cultures but they are incapable of multiplication *in vitro* or *in vivo*.

Rats which receive one or more injections of the living culture acquire a solid immunity which becomes apparent in about 10 days.

The immunity produced by the living culture is lasting and probably persists through life.

The immunity induced by the living vaccine is probably efficacious against the natural transmission through the flea and louse.

The immunity is not due to soluble products, since plasmolyzed and filtered cultures are inert.

This successful immunization against *Tr. lewisi* by means of a living vaccine renders it probable that like results may be obtained with the more strictly pathogenic trypanosomes. It may be added that encouraging results have already been obtained with *Tr. brucei*.

THE INFLUENCE OF CERTAIN OXIDIZING AGENTS (SODIUM IODOSOBENZOATE AND SODIUM IODOXYBENZOATE) ON PHAGOCYTOSIS.*

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Sodium iodosobenzoate and sodium iodoxybenzoate were first prepared by Victor Meyer[1] and his co-workers. They are made from iodbenzoic acid by oxidation. Iodbenzoic acid is made from anthranilic acid (ortho-amido benzoic acid) by treating it with sodium nitrite and potassium iodid in acid solution:

$$C_6H_4\left\langle\begin{matrix}NH_2\\ COOH\end{matrix}\right. \rightarrow C_6H_4\left\langle\begin{matrix}I\\ COOH\end{matrix}\right.$$

Iodosobenzoic acid is prepared by oxidation of ortho-iodbenzoic acid with potassium permanganate in acid solution. This acid has a melting-point of 226°, and crystallizes in large leaves or in needles from dilute solution:

$$C_6H_4\left\langle\begin{matrix}I\\ COOH\end{matrix}\right. + O \rightarrow C_6H_4\left\langle\begin{matrix}I=O\\ COOH\end{matrix}\right.$$

Ostwald[2] demonstrated that this substance is one of the weakest of acids, its dissociation constant being less than that for carbonic acid:

K for iodbenzoic acid.......0.132
K for iodosobenzoic acid....0.00006
K for carbonic acid 0.0013

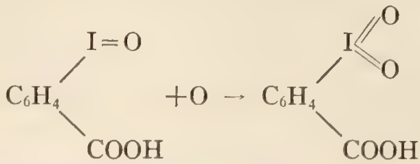
The above property is made use of in the purification of iodosobenzoic acid, by precipitation from solution in sodium hydroxid with carbonic acid.

* Received for publication October 1, 1912.

[1] Meyer and Wachter, *Berichte d. d. chem. Ges.*, 1892, 25, p. 2632; Askenasy and Meyer, *ibid.*, 1893, 26, p. 1354; Hartmann and Meyer, *ibid.*, 1893, 26, p. 1727; *ibid.*, 1894, 27, p. 1600.

[2] *Ibid.*, 1893, 26, p. 1359.

Iodoxybenzoic acid is made from iodosobenzoic acid by oxidation with chlorine:



This substance has marked acid properties. It crystallizes in small glistening needles. The preparation of these substances has been discussed by Loevenhart and Grove.[1]

The action of these substances was first studied by Heinz[2] in 1899. Heinz attempted to study the action of nascent iodin in the body by their use. He was, however, dealing with an impure substance, for his preparations were yellow in color.

The pharmacological action of these compounds has been studied by Loevenhart and Grove and by Loevenhart and the author. These investigations demonstrated that sodium iodosobenzoate and sodium iodoxybenzoate oxidize hemoglobin to oxyhemoglobin, and that sodium iodosobenzoate can furnish oxygen for the peroxidase reaction by oxidizing phenolphthalin to phenolphthalein in the presence of blood. The substances cause no destruction of the blood on intravenous injection, but cause a moderate leukocytosis. They cause a fall of blood pressure on intravenous injection chiefly by depression of the vasomotor center.

Sodium iodosobenzoate and iodoxybenzoate, which contain oxygen, cause cessation of respiration, or apnoea, especially in cats. The respiratory center is most sensitive to these substances, next the vasomotor center. This activity must be due to the oxygen combined with the iodin in them, for sodium iodbenzoate has no such effect.

That their action is due to the available oxygen is further demonstrated by the antagonism between hydrocyanic acid and sodium iodosobenzoate in regard to the respiratory center, as demonstrated by Grove and Loevenhart.[3]

[1] *Jour. Pharm. and Exper. Ther.*, 1911, 3, p. 101.

[2] *Virchow's Arch.*, 1899, 155, p. 44.

[3] *Jour. Pharm. and Exper. Ther.*, 1911, 3, p. 131.

During a study of the effect of these substances on animals in infection I[1] made an investigation of their antiseptic and germicidal action. I found that sodium iodosobenzoate and sodium iodoxybenzoate have a marked germicidal action toward the colon bacillus, the typhoid bacillus, etc., whereas sodium iodbenzoate has very little action. The relative germicidal action toward the colon bacillus was as follows:

Substance	Amount of Oxygen	Concentration Required to Kill	Relative Bactericidal Action
Sodium iodbenzoate.........	None	N/10 (2.7 per cent)	1
Sodium iodosobenzoate.......	6.06 per cent	N/1,000 (0.028 per cent)	100
Sodium iodoxybenzoate......	11.43 per cent	N/2,000 (0.015 per cent)	200

These results indicate that sodium iodosobenzoate and sodium iodoxybenzoate have marked germicidal properties, and that this action must be due to the oxygen present in the molecules. Furthermore, the germicidal action is not destroyed by the presence of serum.

The influence of sodium iodoxybenzoate on the production of antibodies in dogs has been studied by Hektoen.[2] Dogs receiving this substance intravenously produced more antibodies than control animals which received sodium iodbenzoate or no injection at all. This suggests a relationship between antibody production and oxidative processes.

Recently, Amberg and Knox[3] have studied the action of iodoxybenzoate of sodium on the local allergic reaction. They found that it diminishes the intensity of the intracutaneous reaction when given intravenously in rabbits. Iodbenzoic acid does not have this effect. Given intravenously in a sensitized dog it does not interfere with the fall of blood pressure on re-injection of serum. Hence it does not influence the mechanism of the allergic reaction as such, but acts on the inflammatory processes due to a product of the allergic reaction. These inflammatory processes are, on the other hand, favored by sodium cyanide. Because of these interesting properties of sodium iodosobenzoate and sodium iodoxybenzoate, especially the fact that they contain physiologically available

[1] *Ibid.*, p. 145.

[2] *Proc. Chicago Path. Soc.*, 1911, 8, p. 138.

[3] *Jour. Pharm. and Exper. Ther.*, 1912, 3, p. 223.

oxygen, appear to stimulate the production of antibodies, and at the same time have marked germicidal properties, I have studied their action on phagocytosis *in vitro*.

Equal parts of human serum, the substance studied, washed human leukocytes, and suspension of the organism used were drawn up into capillary pipettes, mixed, and incubated at 37° C. for 20 minutes. Smears were then made, allowed to dry in the air, and stained with methyl thionin. Counts were made of the number of bacteria in at least 100 leukocytes from each smear. The same suspension of organisms as well as leukocytes and serum were used throughout each series, the dilution of the substance studied being varied. These dilutions were made in sterile physiological salt solution.

1. SODIUM IODOXYBENZOATE AND STAPHYLOCOCCUS AUREUS.

Mixtures	Phagocytic Index
Serum + salt solution + leukocytes + staphylococcus	1.8
" + N/20 iodoxybenzoate + " + "	2.5
" + N/50 " + " + "	5.6
" + N/100 " + " + "	3.7
" + N/500 " + " + "	2.8
" + N/2,000 " + " + "	3.0

2. SODIUM IODOXYBENZOATE AND STREPTOCOCCUS PYOGENES.

Mixtures	Phagocytic Index
Salt sol. + salt solution + leukocytes + streptococcus	0.81
" + N/50 iodoxybenzoate + " + "	0.82
Serum + salt solution + " + "	3.5
" + N/20 iodoxybenzoate + " + "	3.5
" + N/50 " + " + "	6.4
" + N/500 " + " + "	5.5
" + N/2,000 " + " + "	5.9

3. SODIUM IODOXYBENZOATE AND PNEUMOCOCCUS.

Mixtures	Phagocytic Index
Serum + salt solution + leukocytes + pneumococcus	2.0
" + N/50 iodoxybenzoate + " + "	2.3
" + N/500 " + " + "	2.4
" + N/2,000 " + " + "	2.1

From these results it is seen that sodium iodoxybenzoate has a marked stimulating action on the phagocytosis of staphylococcus

and of streptococcus *in vitro*. This action is not shown by sodium iodbenzoate, which contains no oxygen. The stimulating action is exerted only in the presence of serum, hence the opsonin of the serum is probably rendered more active by the oxygen-containing substances.

The study of the phagocytic index with pneumococcus shows practically no action at all. Dr. E. C. Rosenow tested these substances for their germicidal action toward pneumococcus and found that they have no germicidal action on this organism and instead preserve the cocci from disintegration for a longer time. Did these substances act on the leukocytes, the phagocytosis of pneumococcus should also be stimulated.

These results seem to indicate that the germicidal action of the substances is in some way related to their stimulating effect on phagocytosis, and that both of these properties are the result of the presence in the molecule of physiologically active oxygen attached to the iodin atom. These substances are the only ones thus far known which have a marked germicidal action and at the same time stimulate phagocytosis. That the oxygen is the important factor in the effect on phagocytosis is also indicated by the fact that the cyanides which have an antagonistic action by hindering oxidation also cause marked depression of phagocytosis.

4. POTASSIUM CYANIDE AND STREPTOCOCCUS.

Mixtures	Phagocytic Index
Serum + salt solution + leukocytes + streptococcus	1.74
Salt sol. + " + " + "	0.04
Serum + KCN m/8 + " + "	0.04
" + " m/16 + " + "	0.36
" + " m/32 + " + "	0.32
" + " m/64 + " + "	0.94
" + " m/128 + " + "	1.38
" + " m/256 + " + "	1.68
" + salt solution + " + "	1.78

Since the sodium iodoso- and iodoxybenzoates are able to stimulate the production of antibodies and cause a leukocytosis and can stimulate phagocytosis *in vitro*, they may be of some value in infections.

CONCLUSIONS.

1. Sodium iodoxybenzoate, which contains 11.43 per cent of physiologically active oxygen, has a markedly stimulating action on the phagocytosis of streptococcus and staphylococcus by human leukocytes in the presence of human serum, *in vitro*.

2. Sodium iodbenzoate, which contains no oxygen, has no such effect.

3. This stimulating effect on phagocytosis is related to its germicidal action, which is also dependent on the oxygen combined with the iodin in the molecule.

4. The opsonin of the serum is probably rendered more active by the oxygen-containing substance, for the stimulating effect is exerted only in the presence of serum.

5. Potassium cyanide, whose action is to depress oxidation, has a markedly depressing effect on phagocytosis.

6. Sodium iodoxybenzoate may be of value in some infections because of its stimulating effect on the production of antibodies and on phagocytosis, as well as its bactericidal action.

7. Furthermore, a relationship is suggested between oxidation and the process of phagocytosis, for a substance which liberates oxygen readily stimulates phagocytosis, whereas a substance which prevents oxidation reduces phagocytosis. It would be interesting to study the effect of oxidation on the immune processes.

THE APPLICATION OF THE COMPLEMENT-FIXATION REACTION TO THE DIPHTHERIA GROUP OF ORGANISMS.*†

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The present work forms the second part of a study of the diphtheria group of organisms. The writer is greatly indebted to Dr. H. T. Karsner for advice and the use of the facilities of the experimental division of the laboratory.

The first part of the investigation consisted of a study of the group by the biometrical method.[1] It was found that the group could be divided into two main subgroups, the first comprising the diphtheria bacillus, the second the diphtheroids and Hofmann's bacillus. The second subgroup could be separated further into four species, which are characterized as follows:

B. hoagii, a short, thick bacillus, solid, barred, or wedge-shaped, with indistinct granules; growing very heavily on serum with a salmon-pink color, and fermenting dextrose and saccharose, but not maltose or glycerin.

B. flavidus, a thick, barred bacillus with large granules; having a heavy yellow or yellow-white growth on serum, and acting on dextrose, glycerin, and maltose, but not on saccharose.

B. xerosis, resembling *B. flavidus* morphologically, but giving a meager white growth on serum. It acidifies dextrose, and usually both maltose and saccharose.

B. hofmanni, a small, thick, straight bacillus, barred or wedge-shaped, having no granules, and not acting upon dextrose, maltose, glycerin, or saccharose.

Complement fixation in the diphtheria group has been little studied. Lambotte[2] produced strong specific sera in guinea-pigs for the diphtheria bacillus, a "pseudo-diphtheria" bacillus from the

* Received for publication August 3, 1912.

† Aided by a grant from the Rockefeller Institute for Medical Research.

[1] *Jour. Infect. Dis.*, 1912, 11, p. 253.

[2] *Centralbl. f. Bakt.*, 1901, 30, p. 817.

normal human conjunctiva, and also one from a membrane on a fowl's eye. He gives no description of these bacilli in the above-mentioned article. He found that both the anti-diphtheroid sera gave partial fixation with the diphtheria bacillus.

Weill-Halle and Bloch-Michel[1] used as antigen a suspension of mucus from the throat in salt solution, and anti-diphtheritic serum as antibody. Their results were positive in 25 cases of diphtheria (controlled by culture), and negative in 10 cases of non-diphtheric angina, nine cases of measles, and three of scarlatina. The authors recommend the complement-fixation reaction as quicker and surer than the microscopic diagnosis, for the early detection of diphtheria.

Priestley[2] attempted to use the complement-fixation reaction for the differentiation of the diphtheria bacillus from the diphtheroids, but could obtain no results because of the lack of satisfactory antigens, although he used both bacterial extracts and bacterial solutions in weak antiformin.

Kolmer,[3] using bacterial extracts, found reciprocal reactions among different strains of diphtheria bacilli and also between the diphtheria bacillus and the Hofmann organism.

Some work has also been done on the fixation of complement using diphtheria toxin as antigen. Armand-Delille,[4] employing this with anti-diphtheria horse serum, obtained fixation. He found that there were great individual differences in the fixing power of the sera, and that the horses whose sera deviated strongly had shown signs of anaphylaxis during immunization, while those animals whose sera did not deviate had had no "accidents" during treatment. The author also found that there was no relation between the antitoxic and the fixing properties of the sera.

These results were confirmed by Poujol and Delanoe.[5] Cathoire[6] also found that the serum of healthy diphtheria carriers deviated complement in the presence of diphtheria toxin.

[1] *Soc. méd. d. Hôp. de Paris*, 1910, 29, p. 707.

[2] *Proc. Royal Soc. of Med.*, 1911, 5, p. 146.

[3] *Jour. Infect. Dis.*, 1912, 11, p. 44.

[4] *Mém. de la Soc. de Biol.*, 1908, 65, p. 417.

[5] *Ibid.*, 1908, 67, p. 614.

[6] *Compt. rend. Soc. de Biol.*, 1911, 71, p. 315.

In the present experiments the following representative cultures were used for antigens. For the diphtheria bacillus, Park No. 8, the standard antitoxin strain; for *B. hoagii* a strain obtained from Dr. Louis Hoag, and isolated by him from sputum;[1] for *B. flavidus* a culture obtained by Dr. Hoag from the eye; for *B. xerosis* an organism isolated at the Hoagland Laboratory from a septic uterus; and for *B. hofmanni* a culture, "Cameron," obtained by Dr. Anna Williams of the Research Laboratories of the New York Board of Health. A culture "W," also sent by Dr. Williams, was used in addition. This strain, while showing involution forms similar to the Klebs-Loeffler bacillus, also presented characteristics of the Hoag bacillus, i.e., a slightly pink growth on serum, fermentation of saccharose, and absence of action on maltose and glycerin.

In some preliminary work formalized cultures of the bacilli were used as antigens. They were found to be unsatisfactory, however, on account of their marked anti-complementary properties, and bacillary extracts were therefore substituted for them. The method of preparation of these extracts was the same as that employed by Swift and Thoro,[2] with minor changes. The organisms were grown on serum slants in large bottles for four days, this length of time being necessary to secure a sufficient growth of *B. diphtheriae* and *B. xerosis*. The bacillary suspension was washed once. The organisms, after being dried over sulphuric acid, and weighed, were suspended in 10 c.c. of salt solution, containing 0.5 per cent carbolic acid; ground for one hour in an agate mortar with powdered quartz, and shaken for 18 hours in an International Instrument Company centrifuge shaker at about 500 oscillations per minute. A solution containing one milligram of bacterial substance to one cubic centimeter of salt solution was prepared as needed from the concentrated extract.

Specific sera were obtained by the intravenous inoculation of rabbits. Three animals were immunized for each strain, and the strongest serum was used for the experiments. Formalized suspensions of the organisms were used, from six to 10 doses being given at intervals of five to seven days. A number of animals

[1] *Boston Med. and Surg. Jour.*, 1907, 157, p. 10.

[2] *Arch. of Int. Med.*, 1911, 7, p. 24.

succumbed to inoculations with the diphtheria bacillus, so that the treatment had to be conducted very cautiously, beginning with doses of 0.1 c.c. of the suspension. Potent antisera for *B. diphtheriae*, *B. hoagii*, and *B. flavidus* were produced in this way, but no results were obtained with *B. xerosis* and *B. hofmanni*. Immunization with extracts of these two strains was then undertaken, but proved to be equally unsuccessful. The present study is limited, therefore, to the reactions of the three sera, *B. hoagii*, *B. flavidus*, and *B. diphtheriae*, with six antigens.

A rabbit anti-sheep hemolytic system and guinea-pig complement were used.

The method of procedure was in outline as follows:

1. Determination of the strength of the hemolytic serum. The complement was then titrated each day to the serum.

2. Determination of the anti-complementary properties of the antigens.

3. Determination of the fixing dose of each antigen with normal and immune serum.

4. Trial of each immune serum with descending amounts of each antigen, to discover cross-reactions.

The results of the reactions are given in Tables 1, 2, and 3.

From Table 1 it is seen that the serum *B. diphtheriae* gave partial fixation with the homologous antigen in an amount of 0.025 mg. but that it did not react with any of the diphtheroid antigens or with the atypical diphtheria strain "W."

The serum *B. flavidus* (Table 2) fixed completely with its antigen in amounts of 0.025 mg. It also reacted with *B. hoagii*, *B. xerosis*, and the strain "W" at 0.1 mg., and fixed partially with *B. hofmanni* at 0.4 mg. It did not react, however, with the antigen *B. diphtheriae*.

The serum *B. hoagii* fixed partially with its specific antigen in a dose of 0.025 mg. It also fixed with antigen *B. flavidus* and "W" in amounts of 0.05 mg., but did not react with *B. xerosis*, *B. hofmanni*, or *B. diphtheriae*.

TABLE 1.

FIXING POWERS OF ANTIGENS WITH SERUM *B. diphtheriae.*

ANTIGEN	IMMUNE SERUM *B. diphtheriae*	NORMAL SERUM	COMPLEMENT		HEMOLYTIC SERUM	CORPUSCLES 5 PER CENT SUSPENSION		RESULTS	
								Serum *B. diphtheriae*	Normal Serum
B. diphtheriae—				Incubated one-half hour at 37.5° C.			Incubated one-half hour at 37.5° C.		
0.05 mg	0.1 c.c.	0.1 c.c.	2 units		2 units	1 c.c.		Fixation	Hemolysis
0.025 mg	"	"	"		"	"		Partial fixation	"
B. hoagii—	"								
0.1 mg	"	"	"		"	"		Hemolysis	Slight fixation
0.05 mg		"	"		"	"		"	" "
B. flavidus—	"								
0.4 mg	"	"	"		"	"		Fixation	Fixation
0.2 mg	"	"	"		"	"		Slight fixation	Slight fixation
0.1 mg	"	"	"		"	"		Very slight fixation	Very slight fixation
0.05 mg		"	"		"	"		Hemolysis	Hemolysis
B. xerosis—	"								
0.4 mg	"	"	"		"	"		"	"
0.2 mg	"	"	"		"	"		"	"
0.1 mg		"	"		"	"		"	"
B. hofmanni—	"								
0.6 mg	"	"	"		"	"		"	"
0.4 mg		"	"		"	"		"	"
"W"—									
0.2 mg	"	"	"		"	"		Fixation	Fixation
0.1 mg	"	"	"		"	"		Hemolysis	Hemolysis
Controls—									
NaCl 1 c.c.	"	"	"		"	"		"	"
NaCl 1 c.c.	"	"	....		....	"		No hemolysis	No hemolysis

TABLE 2.

FIXING POWERS OF ANTIGENS WITH SERUM *B. flavidus*.

Antigen	Immune Serum *B. flavidus*	Normal Serum	Complement		Hemolytic Serum	Corpuscles 5 Per Cent Suspension		Results: Serum *B. flavidus*	Results: Normal Serum
B. flavidus—									
0.4 mg	0.1 c.c.	0 1 c.c.	2 units	Incubated one-half hour at 37.5° C.	2 units	1 c.c.	Incubated one-half hour at 37.5° C.	Fixation	Fixation
0.2 mg	"	"	"		"	"		"	Slight fixation
0.1 mg	"	"	"		"	"		"	Very slight fixation
0.05 mg	"	"	"		"	"		"	Hemolysis
0.025 mg	"	"	"		"	"		"	"
B. diphtheriae—									
0.4 mg	"	"	"		"	"		Very slight fixation	Very slight fixation
0.2 mg	"	"	"		"	"		Hemolysis	" " "
B. hoagii—									
0.1 mg	"	"	"		"	"		Fixation	Slight fixation
0.05 mg	"	"	"		"	"		Hemolysis	Hemolysis
B. xerosis—									
0.4 mg	"	"	"		"	"		Fixation	"
0.2 mg	"	"	"		"	"		"	"
0.1 mg	"	"	"		"	"		"	"
B. hofmanni—									
0.6 mg	"	"	"		"	"		Partial fixation	"
0.4 mg	"	"	"		"	"		" "	"
0.2 mg	"	"	"		"	"		Hemolysis	"
"W"—									
0.2 mg	"	"	"		"	"		Fixation	Partial fixation
0.1 mg	"	"	"		"	"		"	Slight fixation
0.05 mg	"	"	"		"	"		Hemolysis	Hemolysis
Controls— As in Table 1									

TABLE 3.

FIXING POWERS OF ANTIGENS WITH SERUM *B. hoagii.*

ANTIGEN	IMMUNE SERUM *B. hoagii*	NORMAL SERUM	COMPLEMENT		HEMOLYTIC SERUM	CORPUSCLES 5 PER CENT SUSPENSION		RESULTS	
								Serum *B. hoagii*	Normal Serum
B. hoagii—									
0.1 mg.	0.1 c.c.	0.1 c.c.	2 units	Incubated one-half hour at 37.5° C.	2 units	1 c.c.	Incubated one-half hour at 37.5° C.	Fixation	Partial fixation
0.0 5 mg.	"	"	"		"	"		Partial fixation	Slight fixation
0.025 mg.	"	"	"		"	"		" "	Hemolysis
B. diphtheriae									
0 2 mg.	"	"	"		"	"		Hemolysis	Very slight fixation
0.1 mg.	"	"	"		"	"		"	Hemolysis
B. flavidus—									
0 4 mg.	"	"	"		"	"		Fixation	Fixation
0 2 mg.	"	"	"		"	"		"	Slight fixation
0 1 mg.	"	"	"		"	"		Partial fixation	Hemolysis
0 05 mg.	"	"	"		"	"		" "	"
0 025 mg.	"	"	"		"	"		Hemolysis	"
B. xerosis—									
0 4 mg.	"	"	"		"	"		"	"
0.2 mg.	"	"	"		"	"		"	"
B. hofmanni—									
0.6 mg.	"	"	"		"	"		"	"
0.4 mg.	"	"	"		"	"		"	"
"W"—									
0 2 mg.	"	"	"		"	"		Fixation	Partial fixation
0 1 mg.	"	"	"		"	"		"	Slight fixation
0 05 mg.	"	"	"		"	"		Partial fixation	Hemolysis
Controls— As in Table 1									

CONCLUSIONS.

The conclusions to be drawn from these experiments are:

1. There are no reciprocal complement-fixation reactions between the typical diphtheria bacillus and representatives of the four species of the diphtheroid group.

2. There are cross-reactions, however, within the diphtheroid group. The species *B. hoagii*, *B. flavidus*, and *B. xerosis* are closely related serologically, as well as morphologically and culturally.

3. *B. hofmanni*, although differing entirely in fermentation powers from the diphtheroids, yet is proved by the complement-fixation test to be related to *B. flavidus*, and therefore must be placed definitely in the diphtheroid group.

4. The action of the culture "W," which is certainly closely allied, both morphologically and culturally, to the diphtheria bacillus, and yet reacts with both *B. hoagii* and *B. flavidus* sera, is further evidence that there are connections between the groups of the diphtheria bacillus and of the diphtheroids.

STUDIES ON HOG CHOLERA.*

EXPERIMENTAL HYPERIMMUNIZATION.

Walter E. King and Robert H. Wilson.

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During the period that *B. cholerae suis* was accepted as the causative factor of hog cholera, investigators attempted to attenuate the organism sufficiently to produce a practical vaccine. Chemicals, heat, and various other methods were employed for this purpose, but the results obtained with these vaccines were not entirely satisfactory. In some instances they protected hogs inoculated with cultures of *B. cholerae suis*, but in the field, as a rule, failed to protect against natural infection.

When it became known that the specific cause of cholera was involved in the so-called "filterable virus," attention was turned to its modification for preventive inoculation. Practically all of the experimental methods used in the attenuation of *B. cholerae suis* have been unsuccessfully applied to the virus. Numerous experiments have been conducted in attempting to attenuate the virus by heating. Dorset and Niles[1] have worked along this line with negative results. Graham[2] also reports unfavorable results with similar experiments. Peters[3] claims to have had more or less success with serum virus heated at a temperature of 60° C. for one-half hour. He has inoculated over 16,000 hogs with this attenuated virus and reports good results in many herds. It is the consensus of opinion, however, that this method of attenuation is not reliable for field use, owing to the difficulty in obtaining a uniform product, and the fact that there is a great variation in the susceptibility of hogs to cholera.

In 1908, King[4] published his results of the attempted attenuation of hog cholera virus by passage through animals of other species, particularly the horse. He concluded that the virus under-

* Received for publication October 1, 1912.

[1] *U.S. Live Stock Sanitary Proceedings*, 1911.

[2] *Amer. Vet. Rev.*, 1912.

[3] *U. S. Live Stock Proceedings*, 1911.

[4] *Bull. Kansas Exp. Sta.*, 1908, *No. 157*.

goes some unexplainable modification resulting in an attenuation after several hours' residence in the circulatory system of the horse. He noted that during the first two hours' residence the virus seemed to become activated in some manner. Typical cases of acute cholera were produced in normal hogs by the injection of two to four cubic centimeters of serum drawn from a horse which had received intravenously a quantity of hog cholera virus two hours previously. Serum drawn at a later period than this seemed to be less virulent, the virulence decreasing in proportion to the length of time elapsing after the horse had received the cholera virus.

In 1910, we[1] attempted to determine whether or not "horse serum virus" (serum drawn from a horse within two hours after the injection of hog cholera virus) represents a mere dilution of the infectious agent. Tests were made to determine this point by preparing corresponding dilutions of the same strains of virus in normal horse blood *in vitro* and also in sterile physiologic salt solution. The proportionate dilutions were made by weighing the horse and taking one-fifteenth of the body weight as representing the total amount of blood in the animal. These dilutions were then kept at body temperature for the same period of time as that of the residence of the cholera virus in the circulatory system of the horse. The virulence of each dilution was tested by the injection of normal hogs kept in separate, well isolated pens. As noted, the serum drawn from the horse within two hours after injection with virus produced cases of acute cholera with an average incubation period of seven days, and an average duration of the disease of 15 days. With the same lot of virus in proportionate dilution in normal horse blood and physiologic salt solution *in vitro*, fewer cases of cholera were produced and those which did develop had a longer average incubation period, and were more chronic in nature. These results indicated that horse serum virus does not represent a mere dilution of cholera virus.

[1] *Ibid.*, 1910, *No. 171.*

HYPERIMMUNIZATION WITH HORSE SERUM VIRUS.

I. EXPERIMENTAL.

The fact that serum drawn from a horse one or two hours after it receives an intravenous injection of approximately 150 c.c. of cholera virus, is capable of producing acute cholera when injected in small doses into normal hogs, suggested the possibility of substituting horse serum virus for hog serum virus in the production of Dorset-Niles hyperimmune serum. Assuming that a reliable, potent serum could be produced by this modified method certain advantages would follow:

The cost of production would be less than that involved in the present method. The serum from one horse could be substituted for the blood from a large number of hogs. This would result, not only in greater economy, but in greater convenience and in better regulated manipulations in serum production. Furthermore, more adequate precautions against contamination could be exercised.

We have published the results of some preliminary experiments relative to hyperimmunization with horse serum virus. These results were fairly uniform in character and indicated that the procedure warranted further experimentation. During the past two years the following data have been collected on this subject.

The virus used in the injection of the horses was in every instance secured from cases of acute cholera in which the symptoms and lesions were characteristic. The moribund hogs were bled from the carotid artery under aseptic conditions. The receptacle containing the blood was placed in a refrigerator for 10 or 12 hours to allow the coagulum to harden and contract. The serum was then poured into sterile flasks which were placed in the refrigerator pending the time of injection.

The horses used in the work, three in number, weighed approximately 1,100 pounds each. They were each about nine years old and constitutionally sound. Each was injected and bled four times. The amount of hog cholera serum injected into the horses at a given time varied from 100 to 150 c.c. The injections, in practically every case, were followed by severe reactions, beginning five or 10 minutes after the horse had received the virus. The

chief characteristics of these reactions were increased respiratory and cardiac movements, stimulation of nearly all the body secretions, and marked general depression. The first symptoms usually disappeared in one hour, leaving the horse more or less exhausted for a day or two. An important feature noted in connection with the injection was that the reactions were usually more pronounced after the first injection. A horse may be repeatedly treated with hog cholera virus intravenously for an indefinite period, as the three animals used in this work are at present alive and in good condition. The bleedings were made one-half to three-quarters of an hour after injection, experience showing that serum drawn from the horse at this time is more virulent than that drawn at an earlier

TABLE 1.

VIRULENCY TESTS OF HORSE SERUM.

Hog	Weight	Date	Amount Serum	Horse	Results	
175	46	9/23	4 c.c.	1,313	Symptoms after 3 days; died after 15 days	Typical lesions
196	55	11/15	4 "	1,313	Symptoms after 5 days; died after 19 days	Typical lesions
250	55	2/28	4 "	1,422	Symptoms after 8 days; died after 30 days	Typical lesions
251	48	2/28	4 "	1,422	Symptoms after 10 days; died after 27 days	Typical lesions
278	60	4/22	4 "	1,423	Symptoms after 6 days; died after 20 days	Typical lesions
279	56	4/22	4 "	1,422	Symptoms after 3 days; died after 13 days	Typical lesions
292	80	5/17	4 "	1,423	Symptoms after 5 days; died after 14 days	Typical lesions
293	80	5/20	4 "	1,314	Symptoms after 7 days; died after 14 days	Typical lesions
294	75	5/22	4 "	1,314	Symptoms after 5 days; died after 15 days	Typical lesions
295	95	5/29	4 "	1,422	No reaction	(Natural immune?)
297	95	6/9	4 "	1,422	Symptoms after 5 days; died after 12 days	Typical lesions
301	48	6/26	4 "	1,423	Symptoms after 4 days; died after 22 days	Typical lesions
334	40	12/4	4 "	1,422	Symptoms after 12 days; died after 22 days	Typical lesions
348	50	11/8	4 "	1,422	Symptoms after 6 days; died after 27 days	Typical lesions

or later period. A specially devised apparatus which defibrinates the blood as it is drawn was used in bleeding. About six liters of defibrinated blood were obtained at each bleeding.

The horse serum virus was tested for virulency before being used for experimental hyperimmunization. One or more normal hogs were injected, subcutaneously, each with four cubic centimeters of the serum from the horse under test. These test hogs were kept in well isolated pens and every precaution was observed

to guard against extraneous infection. Table 1 shows the results of these tests. It will be noted that of the 14 cases reported in the table only one failed to contract cholera from the horse serum virus. Three or four of the animals developed chronic cases, but all succumbed to the disease, showing typical lesions. The average incubation period was 6.2 days and the average duration of the disease 20 days.

Thirteen immune hogs received injections of horse serum virus for the purpose of experimental hyperimmunization. The technic employed in the injecting, bleeding, and handling of the serum varied but little from that in use at most of the hog cholera serum laboratories. The intraperitoneal method of injection was used in practically all cases. One-half of one per cent carbolic acid was added to the defibrinated blood as a preservative. Table 2 gives the number of experimental hyperimmune hogs, their weight, method of injection, amount of defibrinated blood injected, and the number of bleedings.

TABLE 2.

EXPERIMENTAL HYPERIMMUNES.

Hog	Weight	First Injection	Injection Amount	Bleedings	Second Injection	Injection Amount	Bleedings
173......	200	Intraperitoneally and intravenously	1,700 c.c.	8	Intraperitoneally	1,600 c.c.	4
174......	175	Intraperitoneally	900 "	8	.....	....	..
177......	230	"	1,600 "	8	Intraperitoneally	1,800 c.c.	3
217......	58	"	1,400 "	..	"	800 "	7
221......	300	"	2,400 "	3	"	2,200 "	11
254......	200	"	2,000 "	..	"	1,000 "	13
255	190	"	1,800 "	..	"	900 "	14
272*A*	140	"	1,460 "	10	.....	....	..
288......	190	"	1,800 "	..	.....	900 c.c.	11
289......	196	"	2,000 "	Died	.....	....	..
290......	185	"	1,800 "	Died	.....	....	..
291......	190	"	1,800 "	..	Intraperitoneally	1,200 c.c.	15
296......	100	"	900 "	10	.....	....	..

Hyperimmune 173.—A Poland China, weight 200 pounds, immunized by the injection of 70 c.c. hyperimmune serum (Mich. Agric. College) and 2 c.c. of virus on September 13, 1910. Fourteen days later it was injected with 1,700 c.c. of $\frac{3}{4}$-hour horse serum from horse 1,314. Of this amount, 520 c.c. were given intravenously and the remainder intraperitoneally. The hog experienced considerable difficulty in breathing and was otherwise depressed for a few hours after receiving the serum. The first bleeding was made on October 20, when 400 c.c. of serum was obtained. The bleedings were continued at intervals of a week until eight bleedings had been made. As the serum from these various bleedings did not protect the test hogs against cholera, it was deemed advisable to give the hog another injection of the horse serum virus to determine whether this would increase the potency of its serum. This second injection consisted of 1,600 c.c. of horse serum virus given intraperitoneally on

February 16, 1911. The next tail bleeding was made on March 21, followed by three others at intervals of one week. The hog was bled to death on April 19, 1,800 c.c. of blood being obtained. Postmortem examination of the hog revealed no pathological conditions resulting from the injection of horse serum virus.

TABLE 3.

POTENCY TESTS OF SERUM FROM HOG 173.

Bleeding	Hog	Weight	Date	Material Injected	Results	
1st	183	56	10/20	25 c.c. serum + 2 c.c. virus	Symptoms after 11 days; died after 32 days	Cholera lesions not pron'ced
	184	59	10/20	35 c.c. serum + 2 c.c. virus	Symptoms after 11 days; died after 23 days	Fair cholera lesions
	185	51	10/20	2 c.c. virus	Symptoms after 5 days; died after 13 days	Typical lesions
2d	191	52	10/27	25 c.c. serum + 2 c.c. virus	Symptoms after 18 days; died after 21 days	Typical lesions
3d	191	58	10/27	35 c.c. serum + 2 c.c. virus	Symptoms after 7 days; died after 17 days	Typical lesions
	192	53	10/27	35 c.c. serum + 2 c.c. virus	Symptoms after 10 days; died after 18 days	Typical lesions
	193	65	10/27	2 c.c. virus	Symptoms after 4 days; died after 8 days	Typical lesions
4th	195	53	11/14	25 c.c. serum + 2 c.c. virus	Remained well	
	210	50	11/14	4.6 gm. dried serum + 2 c.c. virus	Symptoms after 7 days; died after 19 days	Typical lesions
	211	55	11/14	6.4 gm. dried serum + 2 c.c. virus	Symptoms after 7 days; died after 21 days	Typical lesions
	212	46	11/14	2 c.c. virus	Symptoms after 6 days; died after 8 days	Typical lesions
6th	228	31	1/25	25 c.c. serum + 1.5 c.c. virus	Symptoms after 12 days; died after 18 days	Typical lesions
	229	40	1/25	35 c.c. serum + 1.5 c.c. virus	Symptoms after 11 days; died after 16 days	Typical lesions
	230	33	1/25	1.5 c.c. virus	Symptoms after 6 days; died after 11 days	Typical lesions
7th	235	50	2/3	35 c.c. serum + 2 c.c. virus	Symptoms after 9 days; died after 18 days	Typical lesions
	236	34	2/3	25 c.c. serum + 2 c.c. virus	Symptoms after 6 days; died after 12 days	Typical lesions
	233	38	2/3	2 c.c. virus	Symptoms after 5 days; died after 18 days	Typical lesions
8th	247	40	2/22	25 c.c. serum + 2 c.c. virus	Symptoms after 4 days; died after 23 days	Fair lesions
1st after 2d injection horse serum	256	50	3/21	50 c.c. serum + 2 c.c. virus	Remained well	
	257	50	3/21	40 c.c. serum + 2 c.c. virus	Died after 19 days	Not cholera
	259	48	3/21	2 c.c. virus	Symptoms after 5 days; died after 9 days	Fair lesions
3d	271	100	4/10	40 c.c. serum + 2 c.c. virus	Remained well	
	273	40	4/10	2 c.c. virus	Symptoms after 6 days; died after 15 days	Typical lesions
Slaughter	283	90	4/28	40 c.c. serum + 2 c.c. virus	Remained well	
	284	88	4/28	30 c.c. serum + 2 c.c. virus	Symptoms after 12 days; died after 30 days	Fair lesions
	286	67	4/28	2 c.c. virus	Symptoms after 7 days; died after 11 days	Typical lesions

The serum from hog 173 in most instances failed to protect the test hogs from cholera. It will be noted, however, that the incubation period and duration of the disease were considerably longer in the serum test hogs than in the controls, indicating that a slight resistance to cholera resulted from the injection of the serum.

Hyperimmune 174.—Yorkshire sow, weight 175 pounds. Immunized by the injection of 70 c.c. hyperimmune serum (Mich. Agric. College) and 2 c.c. of cholera virus. Two weeks later injected intraperitoneally with 850 c.c. of ¾-hour horse serum virus. A slight reaction followed the injection as evidenced by anorexia and listlessness lasting a day. First bleeding on October 19, 22 days after receiving the horse serum. Eight bleedings made at intervals of a week.

TABLE 4.

POTENCY TESTS OF SERUM FROM 174.

Bleeding	Hog	Weight	Date	Material Injected	Results	
1st........	180	58	10/19	25 c.c. serum + 2 c.c. virus	Symptoms after 7 days; died after 17 days	Typical lesions
	181	65	10/19	35 c.c. serum + 2 c.c. virus	Remained well	
	182	62	10/19	2 c.c. virus	Symptoms after 10 days; died after 20 days	Typical lesions
2d........	187	85	10/26	40 c.c. serum + 2 c.c. virus	Symptoms after 20 days; died after 25 days	Typical lesions
	188	62	10/26	30 c.c. serum + 2 c.c. virus	Symptoms after 19 days; died after 24 days	Fair lesions
	189	65	10/26	2 c.c. virus	Symptoms after 5 days; died after 5 days	Typical lesions
3d........	204	45	12/19	25 c.c. serum + 2 c.c. virus	Remained well	
4th.......	205	58	12/19	25 c.c. serum + 2 c.c. virus	Remained well	
5th.......	214	50	1/12	25 c.c. serum + 2 c.c. virus	Remained well	
	215	54	1/12	35 c.c. serum + 2 c.c. virus	Remained well	
	216	49	1/12	2 c.c. virus	Symptoms after 6 days; died after 14 days	Typical lesions
6th.......	222	75	1/24	40 c.c. serum + 2 c.c. virus	Remained well	
	223	58	1/24	25 c.c. serum + 2 c.c. virus	Remained well	
	224	56	1/24	2 c.c. virus	Symptoms after 7 days; died after 12 days	Typical lesions
7th.......	237	45	2/8	25 c.c. serum + 2 c.c. virus	Remained well	
	238	59	2/8	30 c.c. serum + 2 c.c. virus	Remained well	
	239	48	2/8	2 c.c. virus	Symptoms after 6 days; died after 13 days	Typical lesions
	288	190	5/6	75 c.c. serum + 2 c.c. virus	Remained well	
	289	196	5/6	75 c.c. serum + 2 c.c. virus	Remained well	
Slaughter..	290	185	5/12	75 c.c. serum + 2 c.c virus	Remained well	
	291	190	5/12	75 c.c. serum + 2 c.c. virus	Remained well	
	286	68	4/28	2 c.c. virus	Symptoms after 7 days; died after 11 days	Typical lesions

With the exception of the first two bleedings, the serum from hog 174 afforded ample protection to the test hogs.

Hyperimmune 177.—A Poland China, weight 230 pounds, received immunizing treatment October 4, consisting of 55 c.c. of hyperimmune serum (Mich. Agric. College) and 2 c.c. of virus. Twenty-seven days later was given intraperitoneally 1,600 c.c. of ¾-hour horse serum virus. First bleeding made November 8. The serum from the first eight bleedings did not prove to be sufficiently potent for practical purposes, and the hog was given a second injection of horse serum February 21, consisting of 1,600 c.c. of serum. Bleedings resumed one month later. Slaughter bleeding April 26.

TABLE 5.

POTENCY TESTS OF SERUM FROM 177.

Bleeding	Hog	Weight	Date	Material Injected	Results	
1st	194	55	11/9	25 c.c. serum + 2 c.c. virus	Symptoms after 7 days; died after 21 days	Slight lesions
2d	197	49	11/16	25 c.c. serum + 2 c.c. virus	Symptoms after 7 days; died after 19 days	Typical lesions
3d	207	46	12/23	25 c.c. serum + 2 c.c. virus	Remained well	
	208	51	12/23	35 c.c. serum + 2 c.c. virus	Symptoms after 8 days; died after 22 days	Fair lesions
	209	43	12/23	2 c.c. virus	Symptoms after 7 days; died after 17 days	Typical lesions
5th	217	58	1/16	35 c.c. serum + 2 c.c. virus	Remained well	
	218	50	1/16	25 c.c. serum + 2 c.c. virus	Remained well	
	219	51	1/16	2 c.c. virus	Symptoms after 5 days; died after 10 days	Typical lesions
6th	225	35	1/24	25 c.c. serum + 2 c.c. virus	Symptoms after 11 days; died after 23 days	Typical lesions
	226	55	1/24	35 c.c. serum + 2 c.c. virus	Remained well	
	227	38	1/24	15 c.c. virus	Symptoms after 5 days; died after 9 days	Typical lesions
7th	232	50	2/3	35 c.c. serum + 2 c.c. virus	Symptoms after 13 days; died after 21 days	Lesions not pronounced
	233	44	2/3	25 c.c. serum + 2 c.c. virus	Symptoms after 16 days; died after 21 days	Lesions not pronounced
	234	45	2/3	2 c.c. virus	Symptoms after 5 days; died after 18 days	Typical lesions
1st after 2d injection horse serum	258	45	3/21	25 c.c. serum + 2 c.c. virus	Symptoms after 24 days; died after 34 days	Typical lesions
	259	38	3/21	2 c.c. virus	Symptoms after 5 days; died after 9 days	Typical lesions
2d	272	64	4/10	30 c.c. serum + 2 c.c. virus	Symptoms after 10 days; died after 28 days	Typical lesions
Slaughter	285	52	4/28	30 c.c. serum + 2 c.c. virus	Remained well	
	286	85	4/28	40 c.c. serum + 2 c.c. virus	Remained well	
	287	67	4/28	2 c.c. virus	Symptoms after 8 days; died after 12 days	Typical lesions

These experiments indicate that the serum from 177 was lacking in protective properties. The second injection of horse serum apparently failed to increase the potency to any extent.

Hyperimmune 217.—Yorkshire, weight 58 pounds, immunized January 16, with 35 c.c. of hyperimmune serum (175, 5th bleeding) and 2 c.c. virus. On May 3, received 1,400 c.c. of ¾-hour horse serum and 800 c.c. one month later, no bleedings

intervening. The first bleeding was made July 3, and bleedings continued weekly until September 1, when the hog was killed.

TABLE 6.

POTENCY TESTS OF SERUM FROM 217.

Bleeding	Time After Injection	Hog	Weight	Material Injected	Results	
3, 4, 5, 6, 7, slaughter..	40, 47, 54, 61, 68 days..	321	55	35 c.c. serum + 2 c.c. virus	Remained well	
		322	45	25 c.c. serum + 2 c.c. virus	Remained well	
		323	42	2 c.c. virus	Symptoms after 5 days; died after 18 days	Typical lesions

The blood from all the bleedings of 217 was combined and tested collectively, with the result that both hogs which received treatment were sufficiently protected, while the control succumbed to the disease.

Hyperimmune 221.—Poland China, weight 250 pounds. Immunized January 23, with 75 c.c. serum (174, 3d bleeding) and 2 c.c. virus. On February 4, was injected intraperitoneally with 2,600 c.c. of $\frac{3}{4}$-hour horse serum. The first bleeding was made a month later, followed by two others at intervals of a week. A second injection of $\frac{3}{4}$-hour horse serum was given April 22, consisting of 2,200 c.c. Bleedings were resumed one month later and continued weekly for 11 weeks.

TABLE 7.

POTENCY TESTS OF SERUM FROM 221.

Bleeding	Time after Injection	Hog	Weight	Material Injected	Results	
1st.........	33 days....	253	35	25 c.c. serum + 2 c.c. virus	Remained well	
2d.........	43 days....	259	58	40 c.c. serum + 2 c.c. virus	Symptoms after 12 days; died after 23 days	Fair lesions
		260	40	30 c.c. serum + 2 c.c. virus	Symptoms after 11 days; died after 20 days	Typical lesions
		261	36	2 c.c. virus	Symptoms after 5 days; died after 9 days	Fair lesions
3d.........	....	266	75	40 c.c. serum + 2 c.c. virus	Symptoms after 14 days; died after 35 days	Typical lesions
		267	55	30 c.c. serum + 2 c.c. virus	Symptoms after 10 days; died after 30 days	Fair lesions
		268	70	2 c.c. virus	Symptoms after 6 days; died after 13 days	Typical lesions
1st after 2d injection..	30 days	296	99	40 c.c. serum + 2 c.c. virus	Remained well	
5th........	62 days	305	42	40 c.c. serum + 2 c.c. virus	Remained well	
		306	39	30 c.c. serum + 2 c.c. virus	Symptoms after 7 days; died after 26 days	Fair lesions
		307	39	2 c.c. virus	Symptoms after 6 days; died after 19 days	Typical lesions
6th........	67 days	308	135	40 c.c. serum + 2 c.c. virus	Remained well	
7, 8, 9, 10, slaughter..	74, 81, 88, 95	314	125	40 c.c. serum + 2 c.c. virus	Remained well	
		315	50	30 c.c. serum + 2 c.c. virus	Remained well	
		317	125	2 c.c. virus	Severe reaction, recovered	

The potency tests showed that the serum from the first three bleedings from 221 was lacking in protective properties. That the test hogs received a degree of immunity is evidenced by the fact that the incubation period and duration of the disease were more prolonged than was the case with the controls in the same tests. The second injection of horse serum stimulated the formation of additional antisubstances. It will be noted that the blood from the seventh to slaughter bleedings was mixed and a composite test made, owing to the shortage of hogs at the time.

TABLE 8.

POTENCY TESTS OF SERUM FROM 254.

Bleeding	Time after Injection	Hog	Weight	Material Injected	Results	
2d.........	30 days	298	77	40 c.c. serum + 2 c.c. virus	Symptoms after 12 days; died after 22 days	Typical lesions
		299	64	25 c.c. serum + 2 c.c. virus	Symptoms after 13 days; died after 22 days	Typical lesions
		300	60	2 c.c. virus	Symptoms after 8 days; died after 22 days	Typical lesions
3d.......	44 days	309	56	40 c.c. serum + 2 c.c. virus	Symptoms after 8 days; died after 17 days	Typical lesions
		310	58	30 c.c. serum + 2 c.c. virus	Symptoms after 8 days; died after 14 days	Lesions not pron'ced
		313	46	2 c.c. virus	Symptoms after 5 days; died after 12 days	Less pronounced
4, 5, 6, 7, 8, 9	51, 58, 65 72, 77, 84	319	47	35 c.c. serum + 2 c.c. virus	Remained well	
		320	37	25 c.c. serum + 2 c.c. virus	Remained well	
		322	45	2 c.c. virus	Symptoms after 5 days; died after 12 days	Lesions not pron'ced
10, 11, 12, 13, 14, slaughter	91, 98, 105, 112, 119	329	65	40 c.c. serum + 2 c.c. virus	Remained well	
		330	62	30 c.c. serum + 2 c.c. virus	Remained well	
		333	65	2 c.c. virus	Symptoms after 6 days; died after 9 days	Typical lesions

Hyperimmune 254.—Yorkshire sow, weight 200 pounds. Received immunizing treatment March 17, consisting of 75 c.c. serum (hog 174, 5th bleeding) and 2 c.c. virus. On April 12 received intraperitoneally 1,800 c.c. of $\frac{3}{4}$-hour horse serum and 900 c.c. May 16. No ill effects followed these treatments other than a slight soreness, which soon passed off. The first bleeding was made one month after the second injection of horse serum. The hog was bled 15 times, and slaughtered September 22.

The first three bleedings yielded a serum which did not afford protection against cholera. Serum from the later bleedings was sufficiently potent to immunize the test hogs.

Hyperimmune 255.—Yorkshire, weight 190 pounds. Immunized May 17, with 75 c.c. serum (hog 174, 5th bleeding) and 2 c.c. virus. On April 12 and May 16, injected with 1,800 c.c. and 900 c.c., respectively, of $\frac{3}{4}$-hour horse serum. No pronounced reactions were caused by these injections. Bleeding commenced one month after second injection.

TABLE 9.

Potency Tests of Serum from 255.

Bleeding	Time after Injection	Hog	Weight	Material Injected	Results	
2d..........	30 days	302	75	40 c.c. serum + 2 c.c. virus	Remained well	
		303	30	30 c.c. serum + 2 c.c. virus	Symptoms after 8 days; died after 28 days	Lesions not pron'ced
		304	64	2 c.c. virus	Symptoms after 8 days; died after 18 days	Typical lesions
3d..........	46 days	311	62	40 c.c. serum + 2 c.c. virus	Symptoms after 10 days; died after 21 days	Typical lesions
		312	58	30 c.c. serum + 2 c.c. virus	Symptoms after 10 days; died after 19 days	Typical lesions
		313	46	2 c.c. virus	Symptoms after 6 days; died after 13 days	Lesions not pron'ced
4, 6, 7......	53, 67, 74	316	120	45 c.c. serum + 2 c.c. virus	Remained well	
		317	130	45 c.c. serum + 2 c.c. virus	Remained well	
		318	125	2 c.c. virus	Severe reaction, recovered	
8 to 15.....	60, 67, 74, 82, 89, 96, 103	331	62	40 c.c. serum + 2 c.c. virus	Remained well	
		332	55	30 c.c. serum + 2 c.c. virus	Remained well	
		333	65	2 c.c. virus	Symptoms after 6 days; died after 9 days	Typical lesions

Hyperimmune 272A.—A Poland China stag, weight 146 pounds. Vaccinated April 10, with 40 c.c. hyperimmune serum and 2 c.c. virus (hog 177, 3d bleeding). Received but one injection of horse serum (1,500 c.c.), this being May 29. Was not bled until three months later.

TABLE 10.

Potency Tests of Serum from 272*A*.

Bleeding	Time after Injection	Hog	Weight	Material Injected	Results	
1 to 10.....	1st, 63 days, others weekly	339	52	40 c.c. serum + 2 c.c. virus	Remained well	
		340	51	30 c.c. serum + 2 c.c. virus	Remained well	
		341	58	2 c.c. virus	Symptoms after 6 days; died after 16 days	Typical lesions

This experiment shows that the various lots of serum from 272*A*, tested collectively, protected hogs from the virus. This animal received but one treatment of horse serum and was not bled until three months later, suggesting that considerable time must elapse between the injection of horse serum and the first bleeding in order to obtain a more potent serum.

Hyperimmune 288.—Yorkshire sow, weight 190 pounds. Immunized to cholera May 6, with 75 c.c. serum (hog 174, 6th bleeding) and 2 c.c. virus. Injected twice with $\frac{3}{4}$-hour horse serum May 20, 1,800 c.c., and June 22, 900 c.c. Bleedings started one month later.

TABLE 11.

POTENCY TESTS OF SERUM FROM 288.

Bleeding	Time after Injection	Hog	Weight	Material Injected	Results	
1 to 11.....	1st, 34 days	337	72	40 c.c. serum + 2 c.c. virus	Remained well	
	Others weekly	338	56	30 c.c. serum + 2 c.c. virus	Symptoms after 6 days; died after 16 days	Typical lesions
		341	58	2 c.c. virus	Symptoms after 6 days; died after 16 days	Typical lesions

Hyperimmune 291.—Poland China, weight 190 pounds. Immunized May 12, with 75 c.c. serum (hog 221, 4th bleeding) and 2 c.c. virus. Injected May 31, with 1,800 c.c. of $\frac{3}{4}$-hour horse serum and again June 30 with 1,200 c.c. No reaction followed these injections. Bleedings started July 29, and continued at intervals of a week.

TABLE 12.

POTENCY TESTS OF SERUM FROM 291.

Bleeding	Time after Injection	Hog	Weight	Material Injected	Results	
1 to 7......	30 days	345	70	40 c.c. serum + 2 c.c. virus	Symptoms after 5 days; died after 17 days	Lesions of chronic cholera
	Weekly intervals	346	67	30 c.c. serum + 2 c.c. virus	Symptoms after 6 days; died after 21 days	Typical lesions
		347	60	2 c.c. virus	Symptoms after 7 days; died after 22 days	Typical lesions

Only the first seven bleedings were tested in this case. As the table indicates, the serum was not potent.

Hyperimmune 296.—Chester white, stag, weight 100 pounds. Injected May 29, with 40 c.c. serum (hog 221, 4th bleeding) and 2 c.c. cholera virus. One month later received 900 c.c. of $\frac{3}{4}$-hour horse serum. Was not bled until August 1, practically two months after receiving the horse serum.

TABLE 13.

POTENCY TESTS OF SERUM FROM 296.

Bleeding	Time after Injection	Hog	Weight	Material Injected	Results	
1 to 9......	1–32 days	343	64	40 c.c. serum + 2 c.c. virus	Remained well	
	Weekly intervals	344	77	30 c.c. serum + 2 c.c. virus	Remained well	
		347	60	2 c.c. virus	Symptoms after 7 days; died after 22 days	Typical lesions

The serum from the various lots of bleedings proved to be sufficiently potent to protect the test pigs against cholera.

TABLE 14.

TABULATED RESULTS OF EXPERIMENTAL HYPERIMMUNIZATION.

Hog	Results	Potency Established
173	Serum not potent	....
174	Serum potent	60 days
177	Serum potent	80 days
217	Serum potent	47 days
221	Serum potent	65 days
254	Serum potent	60 days
255	Serum potent	68 days
272*A*	Serum potent	80 days
288	Potency of serum below standard	34 days
291	First three bleedings not potent. Others not tested	Not determined
296	Serum potent	62 days

SUMMARY.

The results of these various tests relative to experimental hyperimmunization with horse serum virus suggest the following conclusions:

1. Cholera immune hogs can withstand intraperitoneal injections of large quantities of horse serum virus. Ten cubic centimeters per pound body weight can be injected without anaphylactic effects.

2. Horse serum virus when injected into immune hogs is capable of stimulating the formation of antibodies in the blood of the treated hogs.

3. Blood drawn from a hog at least one month after it has received an injection of horse serum virus is more potent than blood drawn at any earlier period.

4. Two injections (intraperitoneal) of horse serum virus, one month intervening, consisting of 10 c.c. and 5 c.c. per pound weight respectively, appear to produce a more potent blood than that produced by one injection.

5. It requires a larger dose of hyperimmune serum, prepared by this modified method, to protect against the test dose of virus, than is necessary with serum prepared after the original method.

II. FIELD TESTS WITH HYPERIMMUNE SERUM.

In order to determine the efficiency of the serum in immunizing against cholera under natural conditions, a number of field tests were conducted. The serum used in these tests had proven potent

at the laboratory and included that from hogs 174, 177, 217, 221, 254, and 255.

Twenty-nine herds, located in three states, received treatment with the experimental serum. The animals in these several herds afforded conditions which are usually encountered in dealing with an epizoötic of hog cholera. The "serum alone" method of treatment was employed in every case.

The following is a description of each herd, together with the treatment and results.

Herd 1.—This herd, an infected one, was located near Flint, Mich., and at the time of treatment consisted of 270 hogs of various sizes and breeds. The disease had been present in the herd for two weeks, apparently having been introduced by a shipment of hogs received from the Detroit stockyards. Thirty hogs had died and 20 were sick, showing symptoms of cholera at the time of treatment (August 26). Three of the sick hogs were killed and postmortem examination revealed characteristic lesions of the disease. One hundred hogs were treated with the experimental serum (221). Of this number 22 were suckling pigs three weeks old; 52 weighed about 50 pounds each; 15 weighed 150 pounds; and 12 weighed 350 pounds each. The doses were 5, 25, 50, and 75 c.c., respectively. Thirty-three hogs were left untreated to serve as controls.

The final result of this experiment shows that 29 per cent of the treated hogs and 84.8 per cent of the controls died. Considering the fact that the herd was badly infected at the time of treatment the outcome of the experiment was quite satisfactory. No attempt was made to isolate the animals in any way during the experiment.

Herd 2.—This herd was located a few miles from Detroit, Mich. It consisted of 15 hogs at the time that the disease appeared. Two weeks later the number was reduced to seven. The disease was prevalent on adjoining farms. Six of the hogs weighing 50 pounds each were treated with 40 c.c. of serum from experimental hyperimmune 221. The remaining shoat was left untreated as a control.

The control and one of the treated hogs died about a week after the treatment. The other five treated hogs remained well.

Herd 3.—This herd was located in northeastern Kansas, in a vicinity where cholera had been prevalent for several months. It consisted originally of 38 hogs, but six of them had already contracted the disease and died. The remaining number had every chance for exposure, as they were in same pen with those which died. Fifteen 30-pound shoats and one 400-pound sow received treatment, consisting of 40 c.c. and 75 c.c., respectively. Three or four of the treated hogs showed slight symptoms of cholera when injected. Sixteen hogs were not treated. Serum 217 was used on this herd. The last report of the experiment shows that 93.7 per cent of the treated hogs and 100 per cent of the controls succumbed. In this experiment the serum had little effect in checking the disease.

Herd 4.—This herd was located one mile from herd 3. Cholera had been present in this herd for two weeks during which time 40 hogs died of the disease. Of the 27 remaining hogs, 19 weighing approximately 70 pounds each were given injections of

the serum (217), the dose administered being 40 c.c. One of these hogs as well as one of the eight controls showed evidence of infection on the day of injection. The final report of this test showed that 42.2 per cent of the treated hogs and 87.5 per cent of the controls died. The serum evidently gave some protection.

Herd 5.—This herd was located one-half mile from herd 4. It consisted of 61 pure-bred Poland Chinas of various ages. The herd had escaped cholera, which was present on adjoining farms. Thirty-five hogs, 16 weighing 75 pounds; three weighing 400 pounds; and 16 pigs three weeks old received treatment (serum 254), the remainder of the herd (26) serving as controls. Cholera entered the herd soon after the treatment, causing the death of 11.4 per cent of the treated hogs and 34.6 per cent of the controls.

Herd 6.—This herd was situated in the same neighborhood as the three preceding herds and though free from disease was in close proximity to it. Fifty-seven shoats were injected, each with 40 c.c. serum (255). Forty-one were left untreated as controls. Reports show that this herd escaped cholera, both treated and untreated hogs remaining in normal condition.

Herd 7.—Non-infected. Herd consisted of 42 shoats, average weight 70 pounds. Owner had visited infected herd on nearby farm and did not exercise any precaution against carrying the disease to his herd. Twenty-eight of the 42 hogs received each 40 c.c. of serum (255). Two weeks later one of the controls died, showing lesions of cholera. Three others were sick at the time, and were destroyed. The treated hogs were in a healthy condition, but owner sold them before completion of the experiment.

Herd 8.—This herd consisted of 76 hogs, mostly spring shoats, weighing 60 pounds, which were apparently in healthy condition. Disease present on farm two miles distant. Thirty-eight were treated with serum (225) receiving 40 c.c. each. The owner reports that the herd is still free from cholera.

Herd 9.—This herd consisted of 85 hogs. They had access to water that flowed through badly infected farms, but at the time of treatment they were free from cholera. Forty-two 50-pound shoats received 40 c.c. serum (217); 43 serving as controls. When the farm was visited one month later, all hogs were in good condition. It was reported later that this herd escaped the disease.

Herd 10.—This was an infected herd consisting of 125, 60-pound shoats, three of which were showing symptoms of cholera. Serum (254) was administered to 63. Two weeks later 15.8 per cent of the treated hogs and 19.3 per cent of the controls had died. Owner sold the remainder before definite results could be obtained.

Herd 11.—This was a non-infected herd located one-half mile from cholera. Of the 48 hogs in the herd 29 received each 40 c.c. serum (217 and 234). When hogs reached marketable age, they were sold, the entire herd having escaped cholera.

Herd 12.—This herd consisted of 32 head, 5 sows and 27 shoats. They seemed to be in good health, although across the road, 40 hogs had died from cholera during the previous month. Three sows were treated each with 60 c.c. serum (217 and 255) and 20 shoats each with 35 c.c. of the same lot. Three weeks later 39.1 per cent of the treated hogs and 88.8 per cent of the untreated had died. The disease appeared in the herd a few days after treatment, six of the controls dying before any of the treated ones showed evidence of cholera.

Herd 13.—This herd located one mile from herd 12 was free from cholera at the time of treatment. Hogs on an adjoining farm were dying of the disease at the rate

of 10 per day. Sixty hogs in herd 13 were injected with the experimental serum (255), leaving 77 as controls. Neither the treated hogs nor the controls became infected while they were under observation.

Herd 14.—This was a non-infected herd consisting of twenty-two 60-pound shoats. There was no cholera within a radius of two miles. Sixteen of the 22 hogs received an injection of the serum (255). The entire herd remained well.

Herd 15.—This herd was located two miles from herd 14. Cholera had been present in this herd for three weeks, and of the original number of hogs (165) only 70 survived and practically all of these showed evidence of infection. Seventeen hogs, four sows, and 13 shoats received treatment, with a dosage of 60 c.c. for the former and 40 c.c. for the latter (serum 173). Twenty-eight animals which showed no symptoms were reserved as controls. One month later 5.9 per cent of the treated hogs and all of the controls had died. The serum proved of considerable value in this herd.

Herd 16.—In this herd 17 remained, out of a total number of 80. Infection had been on the premises for one month. Thirteen of the 17 were given 40 c.c. each of serum from experimental hyperimmune 174. The latest report from this herd shows that both treated and untreated hogs are well.

Herd 17.—This herd consisted of 30 hogs, three of which were sick at the time. The well hogs were placed in new pens and 19 of them (shoats weighing from 30 to 75 pounds) were injected with serum (217). Five weeks later 47.3 per cent of the treated hogs were dead and 45.4 per cent of the controls. Most of these hogs died during the week following the injection, indicating that they were developing the disease when treated.

Herd 18.—This herd was located four miles from cholera, but the owner intended to transfer the hogs to an infected farm four weeks later. Twenty-four were treated with serum (217), 45 c.c. being administered to each, and six were kept as controls. A report from this herd six weeks later shows that all of the controls and one of the treated hogs succumbed to cholera.

Herd 19.—This was a healthy herd of 65 hogs. Proximity of disease, one mile. Fifty-one were treated with serum (173 and 177). Of this number 48 were 60-pound shoats and three were sows weighing about 300 pounds each. Serum injected in amounts of 40 c.c. and 75 c.c. Both treated and untreated hogs remained well.

Herd 20.—These hogs were the property of a cattle feeder and had been purchased from neighborhoods where cholera was prevalent. At the time of treatment they appeared to be healthy. Twenty-nine of the 38 hogs received treatment, which consisted of an injection of 40 c.c. of serum (177 and 217). The final results of this experiment show that 88 per cent of the controls and 51 per cent of the treated hogs had died.

Herd 21.—This herd consisted of 16 shoats, averaging in weight 50 pounds. Twelve of these were treated each with 40 c.c. serum (173). These hogs as well as the controls withstood an infection which was present on adjoining farm.

Herd 22.—In this herd of eight hogs only three were treated, two of which had a temperature of 104.5 at the time of treatment. Both of these hogs died. The other treated hog and two controls survived. Serum used, 254. Dosage 40 c.c.

Herd 23.—Infection not present on farm. Treated 24 hogs, leaving three as controls. Serum 254 was used. This entire herd remained well as long as observation continued.

Herd 24.—Five hogs were treated with serum 253 and 255 in doses of 40 c.c. Two controls. Latest report indicates that cholera had not appeared in the herd.

Herd 25.—In this healthy herd of 14 head, 13 received treatment with serum 255. The disease was present on neighboring farm. A report from this farm six weeks later showed that all hogs were healthy.

Herd 26.—This herd consisted of three 50-pound shoats. Two were injected with serum (254) in a dose of 40 c.c. each. Final result, control dead and both treated hogs well.

Herd 27.—Fifteen hogs in herd. Two died a few days previous to the beginning of the experiment. Ten were injected with 35 c.c. of serum (254). Five weeks later the disease had run its course, with the result that two of the control hogs and none of the treated died.

Herd 28.—One hog treated, three controls. Treated hog received 60 c.c. of serum (254). The result was that the treated hog remained well and two of the controls died.

Herd 29.—There was an infection one-half mile from this herd. Ten of the hogs were given the serum treatment, a dose of 70 c.c. being administered (221). The latest report shows that cholera has not entered the herd.

TABLE 15.

SUMMARIZED RESULTS OF FIELD WORK.

Condition of Herds	Number of Hogs Treated	Number Died	Percentage	Number Controls	Number Controls Died	Percentage
Not exposed before or after treatment.......	371	0	0	267	0	0
Exposed before treatment and infection evident..	308	99	32.1	191	122	63.8
Exposed, no symptoms before treatment......	87	5	5.7	46	19	41.3
Total..............	766	104	....	504	141	....

A general survey of the field tests, as well as of those conducted at the laboratory, indicates that the experimental serum used in this work possessed protective properties. Of the 29 herds treated, 13 were infected at the beginning of treatment. The disease was of an extremely virulent type in four of these herds, namely numbers 1, 3, 4, and 12. In the infected herds a total of 308 hogs received treatment and 191 served as controls. The final results show that 67.9 per cent of the treated hogs and 36.2 per cent of the controls survived, which points to the fact that the serum had both a prophylactic and curative effect. In the case of the non-infected herds only three of the 16 became exposed after treatment. In the three herds which became exposed to hog cholera after experimental treatment (herds 5, 7, and 18) the average final result

is expressed by a survival of 94.3 per cent of treated hogs and of 58.7 per cent of the controls.

GENERAL CONCLUSIONS.

The practical use of horse serum virus in the preparation of hyperimmune serum is open to question. The method, in so far as our results show, has two disadvantages:

1. The animals used for hyperimmunization must be kept under treatment several weeks longer than when treated by the original method.

2. A larger dose of the serum, as compared with that which is recommended by those engaged in preparing hyperimmune serum according to the original formula, must be administered.

This work has resulted in further experimental evidence that horse serum virus represents an activated hog cholera virus. It would scarcely appear possible to produce hyperimmune serum as relatively potent as that which has been used in this work, were similar dilutions of the original cholera virus, in physiologic salt solution *in vitro*, used for the purpose of hyperimmunization in substitution for "horse serum virus."

THE PRODUCTION *IN VITRO* IN THE NORMAL BRAIN OF STRUCTURES SIMULATING CERTAIN FORMS OF NEGRI BODIES.*

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The Negri bodies have been interpreted in two ways, by some observers as protozoa, by the majority, however, as degeneration products of nerve cells. In each of these interpretations the bodies are regarded as specific for rabies. Thus far, neither view has been supported by experimental evidence. The following work was therefore undertaken in the hope that some light might be thrown upon the nature of the bodies in question. Although the results are not entirely conclusive, they lend weight to the view of the degenerative origin of at least some of the smaller forms of Negri bodies.

EXPERIMENTAL WORK.

The results of the present investigation, which refer, in brief, to certain morphological changes in brain cells under special conditions of incubation, may be conveniently described in three parts, as they relate (1) to the normal brain, (2) to the normal brain inoculated with rabies virus, after the death of the animal, and (3) to the rabid brain.

Technic.—The technic employed consisted simply in an application to our purposes of Harrison's method of cultivating tissues *in vitro*. Small pieces of the brain of rabbits and guinea-pigs were suspended in hanging drops of blood plasma obtained from the animal from which the brain was to be removed. All portions of the brain were used, but Ammon's horn gave the most satisfactory results in that the ganglion cells in this region developed the most numerous degenerative structures. The culture preparations were incubated at 37.5° C. for varying lengths of time. In each experiment a number of preparations (20 to 50) were

* Received for publication September 20, 1912.

made in order to allow a serial study of the stained cells. An examination was made every two days after incubation was begun, four to 10 slides being used each time. Van Gieson's[1] method for the rapid diagnosis of rabies, as modified by Williams,[2] was used for staining the specimens.

In this connection we wish to record our indebtedness to Dr. Van Gieson for his generous assistance in this study.

I. INCUBATION OF NORMAL BRAIN.

At the beginning of the present study it was decided to attempt first to produce Negri bodies *in vitro* by combining outside the body rabies virus and living brain cells. This accomplished, it seemed to us that a study of the character of the bodies could more easily be undertaken. With this end in view an experiment with virus-injected guinea-pig brain, as described in the next division of this paper, was made, a control, however, being made in which an uninjected normal guinea-pig brain was used.

In examining smears from the bits of tissue in the hanging-drop preparations after incubation we were interested to find in the ganglion cells in *both* series structures practically identical in appearance with certain forms of Negri bodies found in the rabid brain. To exclude the possibility that some error in technic may have been responsible for the findings in the normal control specimens, further experiments were made using pieces of normal brain only. The results of this study will now be briefly described.

Smears made from the pieces of tissue after four to six days' incubation show ganglion cells with well preserved nuclei. The cells are considerably enlarged. If one compares the cells shown in Figs. 1 and 2 it is seen that the increase in size involves both nucleus and cytoplasm. The nuclei stain lightly by Van Gieson's method. The cytoplasm shows numerous round clear spaces which represent fat droplets in the living cell. It should be noted here that a similar intracellular accumulation of fat has been regularly observed in the cultivation *in vitro* of the various body tissues. It probably indicates a condition of disturbed metabolism resulting from the changed conditions of life.

[1] *Proc. N.Y. Path. Soc.*, 1906, 5, p. 83. [2] *Ibid.*, 1905, 5, p. 155.

In the cytoplasm of a certain number of the cells there are found small pink-staining bodies, one of which is shown in Fig. 2. These bodies, which we have called "degenerations," are not distinguishable by the usual methods of staining from the small intracellular unstructured Negri bodies observed with great frequency in the rabid guinea-pig brain. Some of the "degenerations" are surrounded by a blue granular ring, such as is seen in typical Negri bodies. In a few instances, structured forms were also found showing a small blue-staining ring in the center (Fig. 3). These "degenerations" closely resemble the small structured forms of Negri bodies in the rabid brain.

In addition to the two intracellular forms, extracellular unstructured forms were also found. Specimens incubated for a longer time (10 to 20 days) did not show any further changes in the character of the structures.

In one experiment in which the brain of a normal puppy was used, the results were unsatisfactory owing to the rapid degeneration of the ganglion cells in the cultures. Whether this was due to faulty technic or to exceptional delicacy of the brain cells of the dog, it will be necessary for further work to determine. Since the Negri bodies found in the brain of a rabid dog have a more complex structure than those in the guinea-pig, the dog's brain should be better suited to studies such as we have undertaken. Further experiments in this direction are contemplated.

II. INCUBATION OF BRAINS INOCULATED WITH VIRUS.

Two series of experiments were made using brains from freshly killed animals injected *in situ* with rabies virus. In both series normal guinea-pig brains were used.

Series 1.—Street virus; gland extract: The submaxillary glands of a dog dying of rabies (street virus) were extracted under sterile conditions with glycerin. The glycerin was removed by dialysis and the virus concentrated in physiological salt solution by the use of collodion sacs. The virus was injected thoroughly into the substance of the brain by means of a hypodermic syringe. Hanging-drop plasma preparations were made, and incubated as in the preceding set of experiments.

Series 2.—Fixed virus; brain emulsion: The brain of a fixed-virus rabbit was used for making the emulsion. A normal guinea-pig brain was injected as in Series 1, and hanging-drop plasma preparations were made.

In both series the results were identical with those obtained with the normal uninoculated brain; that is, the small intracellular unstructured "degenerations," with an occasional structured form, were found after four to six days' incubation.

III. INCUBATION OF RABID BRAINS.

Two series of experiments were carried out using street and fixed viruses.

Series 1.—Street virus; guinea-pig's brain: Hanging-drop preparations made with tissue from the brains of guinea-pigs dying of street virus were incubated and examined as in preceding experiments.

Stained smears of the brain before incubation showed mostly the small unstructured Negri bodies usually found in the rabid guinea-pig brain. Upon incubation no development of the forms could be observed, and apparently there was no increase in number. It should be noted here that ganglion cells appear to live in the cultures for 10 to 20 days or longer. One preparation, 21 days old, still contained cells that appeared to be living though greatly enlarged. Observed with a 1/12 objective and a No. 4 eye piece, one such cell stretched entirely across the field.

Series 2.—Fixed virus; rabbit brain: Small pieces of the brain of a rabbit dying of fixed virus were incubated and stained as in the preceding experiments. In the incubated material small forms with central structure were somewhat more numerous than in the fresh brain. But since no study has as yet been made of the normal rabbit brain incubated in the same manner, we are unable to say how frequently the unstructured "degenerations," such as were observed in the normal guinea-pig brain, would be found under the same experimental conditions.

IV. INOCULATION OF ANIMALS WITH INCUBATED RABID MATERIAL.

Guinea-pigs were inoculated intracerebrally with the incubated preparations of rabid brains as well as with incubated pieces of

PLATE 3.

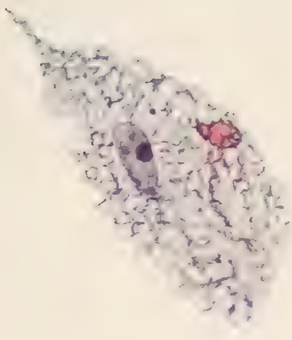

FIG. 1.

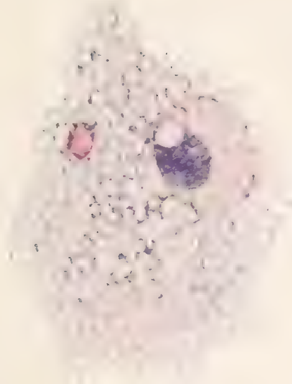

FIG. 2.

FIG. 3.

normal brains previously injected with virus. The periods of incubation of this material were from six to 10 days. There was only one positive result from the inoculations. This was from material from a rabid guinea-pig brain (street virus) incubated for eight days. In this instance, although the virus had lived, there was, in the stained preparations, no evidence that multiplication or development of the Negri bodies had taken place.

SUMMARY.

1. The cells of the normal guinea-pig brain, incubated in blood plasma, showed in their cytoplasm, when stained by Van Gieson's method, small pink-staining bodies surrounded by a blue granular ring, indistinguishable from the unstructured Negri bodies observed with great frequency in the rabid guinea-pig brain. In a few instances these forms contained a central blue-staining ring or point, and closely resembled some of the smaller structured forms of the Negri body.

2. The normal guinea-pig brain inoculated with rabid material, street or fixed virus, incubated in the same manner, showed the same structures.

3. The brains of guinea-pigs dying of street virus, and rabbits dying of fixed virus, incubated in small fragments, gave no development of the Negri bodies in blood plasma, beyond the small structured and unstructured forms, although in one preparation the ganglion cells appeared to be living at the end of 21 days' incubation.

EXPLANATION OF PLATE 3.

FIG. 1.— Ganglion cell containing a small unstructured Negri body: from a rabid guinea-pig brain (street virus).

FIG. 2.— Ganglion cell containing a small unstructured "degeneration" from a piece of normal guinea-pig brain incubated in blood plasma six days.

FIG. 3.— Small structured "degeneration" in a ganglion cell from a bit of normal guinea-pig brain incubated eight days.

All preparations were stained according to Van Gieson's method.

BOVINE INFECTIOUS ABORTION, EPIZOOTIC AMONG GUINEA-PIGS.*

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The statement has frequently been made that, while the bacillus of bovine contagious abortion (*Bacillus abortus*, Bang) will cause abortion in other domestic or laboratory animals when experimentally inoculated, it is not known to be epizootic among any animals except cattle. Abortion of an epizootic nature occurs not infrequently with horses, sheep, and swine. In no instance, however, has such a case been connected with the organism found in cattle. There is a considerable body of evidence to show that the natural infection of animals other than cattle with this organism must be exceedingly rare. However, the ease with which other species of animals may be artificially infected leaves it by no means certain that under the proper conditions natural infection might not occur. In this connection, the following observations are of interest.

In 1908 the Department of Animal Husbandry of this station began some work with infectious abortion. From that time until the end of 1911 a number of guinea-pigs were inoculated from time to time with material from aborting cows. During the year 1911 a number of these animals were inoculated with pure cultures of the bacillus abortus. During this period the space for keeping inoculated animals was very limited. A room in the station piggery had been fitted up as a growing room for such small animals. During a large portion of the year there was no place suitable for inoculated pigs except in this room. The inoculated pigs were kept in wire cages of the Vaughan type. These cages were on legs about six inches high, and were placed in the growing pens so that the healthy pigs could run under the cages. No inoculated pigs were ever returned to the general herd and none of the healthy animals could come in contact with the infected pigs except through the one-eighth inch wire screen on the sides of the cages. However, such an arrangement allowed infected litter and urine to reach the

* Received for publication September 10, 1912.

outside of the cages, and thus to come in contact with the healthy animals.

In January of the present year I undertook some extensive experiments on the diagnosis of bovine infectious abortion,[1] using the agglutination and complement fixation reaction. In this latter test guinea-pig's blood was used for the complement. In the beginning some very puzzling results were obtained in which control tubes containing only complement and antigen showed complete fixation of the complement. It was first believed that there was some trouble with the antigen, and various modifications of this were tried without avail. It was then noticed that this result was obtained only with the serum of certain guinea-pigs. The serum of such pigs was then tested for its ability to agglutinate the abortion bacilli. It was found that the serum of such pigs agglutinated the abortion bacilli very strongly, even in dilutions of 1 to 1,500 or higher. The serum of those guinea-pigs which did not fix the complement likewise did not agglutinate the abortion bacilli. It is thus practically certain that these guinea-pigs had been infected with the abortion bacilli and that for this reason their blood contained abortion amboceptors.

Records have been kept of 43 guinea-pigs killed for complement from this general herd. Of these the blood of 29 showed the presence of abortion amboceptors, while 14, mostly young pigs, gave no evidence of infection. The sera of 12 of the 29 guinea-pigs which showed fixation of the complement were also tested for agglutination. In every case the serum gave a strong agglutination.

To all appearances the herd of guinea-pigs is perfectly healthy and vigorous. We have noticed an occasional pig showing paralysis of the hind quarters but these occur so rarely that no importance is attached to it. However, it is certain that the herd is not multiplying as rapidly as formerly, although no definite data regarding this can be given. If abortions occur in any number, the products are eaten so quickly that they are rarely seen. Under the conditions in which the animals are kept this could very well happen. When pregnant females have been confined we have often observed abortions.

[1] *Ky. Agric. Exp. Sta., Annual Report,* 1912. Bull. No. 166, pp. 301, 366.

Smith and Fabyan[1] have recently pointed out that guinea-pigs inoculated with the Bang bacillus present certain characteristic lesions. One of the more marked pathological changes is the enlargement of the spleen which occurred in 98 per cent of the cases examined by them.

It is thus of some interest to go through our autopsy records and note the condition of this organ in connection with these blood tests. It is a rule of the laboratory to make autopsy records, including the weights of the principal organs, of every animal killed. Unfortunately, on account of the lack of proper facilities, complete records were not kept on all the guinea-pigs killed in the earlier part of this work. Since the first of March, however, the records are fairly complete. A portion of these records with respect to spleen weight are shown in the following table:

Guinea-Pigs Showing a Blood Reaction with Abortion Antigen			Guinea-Pigs Showing no Blood Reaction		
Body Weight, Grams	Sex	Spleen Weight, Grams	Body Weight, Grams	Sex	Spleen Weight, Grams
465	♀	3.50	576	♀	1.20
634	♂	1.75	376	♂	0.60
580	♀	5.50	502	♂	0.65
579	♂	2.50	203	♂	0.75
485	♀	1.03	308	♀	0.64
289	♀	0.75	444	♂	0.35
502	♀	1.50	308	♀	0.40
672	♀	6.00	348	♂	0.40
680	♂	1.75	550	♀	0.90
602	♂	3.10			
Average 548.8		2.74	401.7		0.65

From this table we note that the average spleen weight of the pigs showing a reaction was over four times the average weight of the spleens from non-reacting pigs. On the other hand, the average body weights of the reacting animals was only about one-third greater than that of the non-reacting pigs. This difference in body weights is accounted for by the fact that it was mainly young pigs which did not show a reaction.

With one exception the spleen weight of every guinea-pig showing a reaction was greater than one gram, while of the non-

[1] *Centralbl. f. Bact.*, 1912, Abt. 1. Orig., 61, p. 549; *Jour. Exper. Med.*, 1912, 27, p. 441.

reacting animals only one shows a spleen weight of one gram or over.

It is further noted from this table that the sexes are distributed about equally in the two groups. This shows that the males are as likely to be infected as the females. This point has not always been clearly recognized in studying the disease in cattle.

The results shown in this table are in agreement with the results obtained by Smith and Fabyan on experimentally inoculated guinea-pigs. These results confirm the conclusions drawn from the serum reactions.

In order to furnish definite proof that these lesions and blood reactions are due to infection by the abortion bacillus it is necessary to isolate the organism from guinea-pigs showing these reactions. This has been done in two instances. In one case cultures were obtained from the spleen and liver and in the other from the spleen alone. Cultures made from several other reacting pigs did not show any growth of this organism. I have noted at another place[1] that the abortion organism when first isolated is frequently very difficult to grow under laboratory conditions. This may account for the failure to obtain a culture in every case.

In February, 1912, a number of apparently healthy guinea-pigs two or three weeks old were removed from the general herd and placed in a separate building at some distance from the piggery. During May and June a number of these young pigs were killed for complement. None of these showed any evidence of infection. This would indicate that the strict isolation has prevented the further spread of the infection.

It is believed that these data are of interest in showing that, under certain conditions at least, the bacillus of bovine infectious abortion can become epizootic among animals other than cattle. It opens the possibility that under similar conditions other domestic animals might become infected.

[1] *Loc. cit.*

INTRALEUKOCYTIC BODIES IN SCARLET FEVER.*

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(From the Memorial Institute for Infectious Diseases, Chicago.)

Since Döhle's first article on "Leukocytic Inclusions in Scarlet Fever," a number of workers have investigated the occurrence, significance, and nature of such inclusions but the conclusions derived from these investigations differ very much.

Döhle[1] examined smears of blood fixed by alcohol from 30 cases of scarlet fever and from numerous other diseases, which he does not specify. The smears were stained either with a mixture of Hoppe-Seyler's reagent, water and Michaelis' azure blue, or with an acid solution of orseiles mixed with acid hematoxylin. He also used Pappenheim's stain. By these methods he observed inclusions in the cytoplasm of nearly all neutrophiles almost invariably in scarlet fever. These bodies stained blue with the two first-mentioned stains, and took a pale red with Pappenheim's mixture. Their size and shape varied greatly, some being as large as one-sixth the size of the nucleus, and round or rod-shaped, or oval with one pointed end. The number of bodies per cell varied from one to six. The only cases of scarlet fever in which such bodies did not occur were two examined late in the disease. Only in cases of two other diseases were similar bodies found; viz., two cases of carcinoma and one of pneumonia, in which there was a history of syphilis (the author stating that in this particular instance his slides might have become mixed with those of scarlet fever). Döhle holds that because the bodies stain red with Pappenheim's stain they need not be confused with nuclear fragments; he also finds similar bodies free in the blood.

In a more recent article Döhle[2] describes spiral forms in the cytoplasm of leukocytes, and also in the blood of two cases of scarlet fever. These he believes are spirochaetes and he names them *Spirochaeta scarlatinae*. The bodies which he described in the leukocytes he holds to be fragments of the parasites of both diagnostic and prognostic significance.

Stimulated by Döhle's work, Kretschmer[3] studied some 30 cases of scarlet fever and about 70 controls. At first he used Döhle's method, but soon changed to fixation by methyl alcohol and staining with Manson's stain, because this technic is equally efficient and much simpler. He also tried Giemsa's stain. Kretschmer verified Döhle's results in regard to scarlet fever, finding bodies in all early cases; they usually disappeared by the 8th–10th day, occasionally somewhat later. Döhle and Kretschmer both failed to find such bodies in animals; the latter failed to find any relation between the temperature and the occurrence of the bodies. The other diseases in which he found the bodies, but by no means constantly, were diphtheria, tuberculosis, and pneumonia. They were not found in leukocytes from normal persons or in cases of measles.

Nicoll and Williams[4] used Kretschmer's technic, and also Giemsa's stain over night. In 51 cases of scarlet fever in which the patients had been sick more than eight days the bodies were present in all except six cases. Bodies were also found

* Received for publication October 1, 1912.

[1] *Centralbl. f. Bakt.*, I, Orig., 1912, 61, p. 63.

[2] *Centralbl. f. Bakt.*, I, Orig., 1912, 65, p. 57.

[3] *Berl. klin. Wchnschr.*, 1912, 49, p. 499.

[4] *Arch. Ped.*, 1912, 29, p. 350.

in one of 12 cases of measles—a doubtful case—and in one case of erysipelas and one of pneumonia. Nicoll and Williams believe strongly in the diagnostic value of the bodies. In another article, Nicoll[1] reports the results of the study of 115 cases of scarlet fever; in 16 only were the bodies not found. One of the patients had been sick four days and the diagnosis was questionable, two had been sick five days, and the remainder were sick for a longer period. He found them also in erysipelas, typhoid fever, and sepsis.

Ahmed[2] does not regard the bodies as peculiar to scarlet fever because he finds them in tuberculosis, erysipelas, and measles. He holds that they have some relation to fever and that they may be nuclear fragments or degenerated cytoplasm.

Kolmer's[3] results in 216 cases of scarlet fever agree with those of Döhle and others. He found them in the majority of 50 cases of diphtheria, in erysipelas, sepsis, pneumonia, and empyema. He failed to find them in measles (12 cases) and in a number of other diseases including varicella, pelvic diseases, pericarditis, goiter, furunculosis, eczema, gonorrhea, bronchopneumonia, German measles, mastoiditis, gastroenteritis leukemia, and pernicious anemia. Kolmer concludes that the occurrence of the bodies is connected with streptococcal infections, and that they are of value in differentiating scarlet fever from rötheln, measles, and gastrointestinal rashes. He believes them to be derived from the cytoplasm of the cells.

The results obtained so far may be summarized as follows: It is agreed that certain bodies are present in the leukocytes in the early stages of scarlet fever, which are not found in normal blood; but authorities differ as to their occurrence in other diseases, as to their diagnostic significance, and as to their nature.

In the study now reported the blood was drawn from the lobe of the ear, smears made and fixed with methyl alcohol. Smears were also fixed by formalin, heat, alcohol, and ether, for the purpose of comparison. Manson's stain was used for the most part, but methylene blue, Delafield's hematoxylin, Leishman's stain, Pappenheim's stain, and carbol-thionin were also used. Cases of scarlet fever, erysipelas, and measles were studied each day for a longer or shorter time after their entrance into the hospital, and in each instance at least 100 neutrophiles were examined. Twenty-five cases of scarlet fever were examined. In six of these the result was practically negative, that is, very few leukocytes with bodies in their interiors were found. In one of the negative cases the illness which was exceptionally mild had lasted only five days; in the others the examinations were made after the febrile stage had passed. In the other 17 cases there was no difficulty in

[1] *Arch. Ped.*, 1912, 29, p. 416.

[2] *Berl. klin. Wchnschr.*, 1912, 46, p. 1232.

[3] *Amer. Jour. Dis. of Child.*, 1912, 4, p. 1.

finding the bodies. In one case 68 per cent of the neutrophiles contained the bodies; in most of the others the percentage ran between 25 to 40. In making the counts only cells containing definite and distinct bodies were counted as positive. By daily examination, it soon became apparent that the percentage of leukocytes with bodies varied from day to day, and that there are fairly constant relationships in each case in this respect. Thus the highest counts were found on the fifth day in most cases, the average being slightly lower on the fourth day. Chart 1, which illustrates this point, shows that the leukocytes with inclusions increased in number until the fifth day and then gradually diminished, none being found on the ninth day. In most cases of scarlet fever the leukocytes became practically free from bodies between the seventh and 10th days of the disease. The bodies were found to be most numerous when the clinical symptoms were most marked. As shown in Chart 2, the temperature curve corresponds quite closely to that of the leukocytes

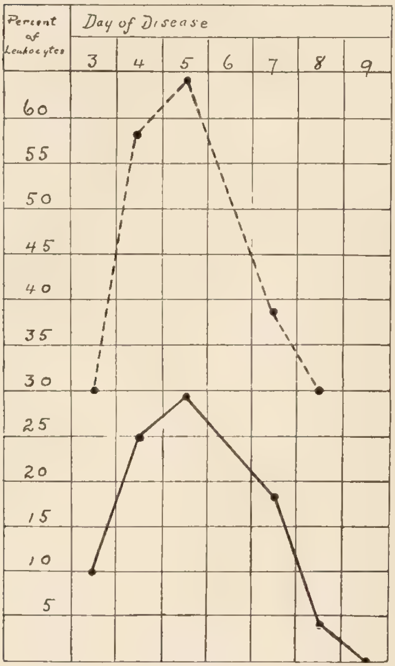

CHART 1.—Percentage of leukocytes containing free bodies and those containing fragments attached to nuclei, in a case of scarlet fever.
——— = leukocytes with free bodies.
- - - - - = leukocytes with attached fragments.

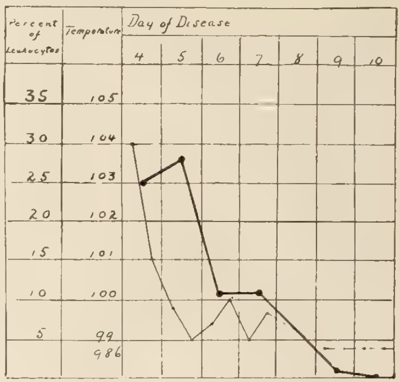

CHART 2.—The relation of free bodies in leukocytes to temperature, in a case of scarlet fever.
——— = leukocytes with free bodies.
——— = temperature.

with inclusions, anticipating the latter by about a day. When the temperature becomes normal the inclusions practically always disappear in from 24 to 48 hours, and when fever again reappears the bodies also return.

Seven cases of measles were examined, two being afebrile at the time, and in one of these 10 per cent, and in the other 3 per cent of the leukocytes contained bodies. In the other five cases from 10 to 24 per cent of the leukocytes were positive, the highest percentage being obtained in the case of a child with hemorrhagic measles with high temperature (see Fig. 1).

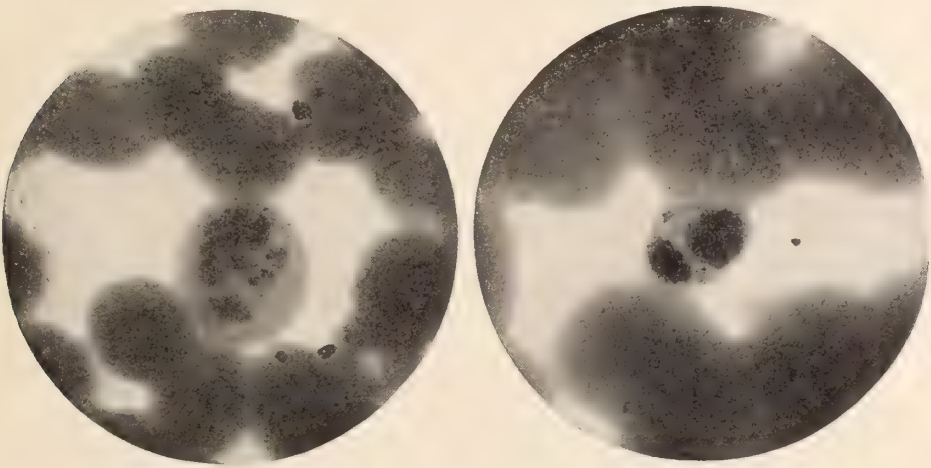

FIG. 1.—Intraleukocytic bodies in measles. × 1000.

The same relation between the intraleukocytic bodies and fever was also found in erysipelas of which five cases were studied. Two of these were afebrile and practically free from bodies. The highest percentage in erysipelas was 60. This occurred in the case of an old man on the day on which his temperature was 104°.

In all these cases the leukocytes showed partially detached nuclear fragments similar in general outline to the free bodies, and also irregular projections or stumps from the nuclei, as if fragments had been torn off. Indistinct spiral bodies similar to those described by Döhle as *Spirochaeta scarlatinae* were found in measles and erysipelas as well as in scarlet fever.

The other cases examined may be divided into febrile, and afebrile.

All of the fever cases studied gave positive results; viz., two cases of diphtheria, two of fever due to antitoxin, two of gastrointestinal fever, two of empyema, a case each of brain tumor and of neuritis, two cases of malaria, two of tuberculosis, and four cases of typhoid fever.

In four normal individuals examination of 100 cells in each person showed that from 2 to 4 per cent of the leukocytes contained bodies similar to those in scarlet fever. When the blood was subjected to a vigorous shaking in citrate solution, and afterward washed, the number of bodies was greatly increased so that the percentages rose to from 12 to 20, and at the same time the number of leukocytes with irregular nuclear projections were increased proportionately.

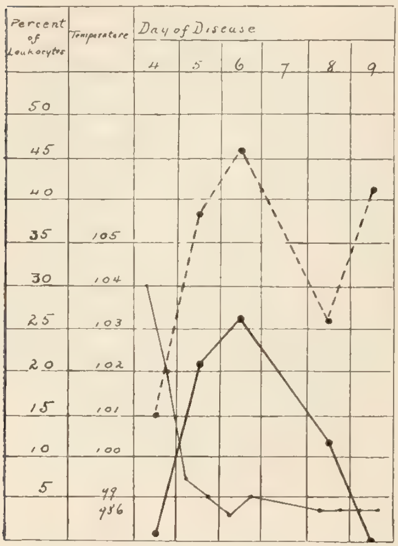

CHART 3.—The relation between percentages of leukocytes containing bodies, and those containing fragments attached to nuclei, in a case of measles.

——— =leukocytes with free bodies.

- - - - - =leukocytes with attached fragments.

——— =temperature.

Bodies were also found in the leukocytes in three cases of local suppuration without fever, in a case of myelogenous leukemia, and a case of rickets.

In attempting to arrive at some conclusion in regard to the nature and origin of these bodies, I would suggest that their size and shape and also their reactions with staining agents indicate strongly that the majority of them are nuclear fragments. Similarly shaped bodies partially detached from the nucleus are found regularly. In scarlet fever, measles, and erysipelas the percentage of leukocytes containing partially detached fragments is a little larger than the percentage containing free bodies, both percentage curves

maintaining, however, the same relation to the temperature curve (Chart 3). In fact, so far as my actual observations go, from 12 to 24 per cent of the leukocytes of blood from other sources than patients with scarlet fever, measles, and erysipelas, both in health and disease, show these attached nuclear fragments. I have stated already that by rough manipulation of normal leukocytes the number of free intraleukocytic bodies may be increased very definitely, and that at the same time there is an increase in the number of partially attached masses. The width of the pedicle may vary from that of the slenderest thread to $2\,\mu$ or more. Pointed projections from the main nucleus are seen often, and suggest that fragments have been detached. Often free bodies lie in juxtaposition to such stumps. The fact that free bodies within leukocytes are colored red with Pappenheim's stain does not prove that they are not of nuclear origin, because it is well known that chromatin when separated from the nucleus soon changes in its staining reactions. Of course, it is not my intention to claim that all the bodies within the leukocytes in scarlet fever are nuclear fragments. Some may be parasites or parasitic remnants, others blood platelets, and still others of cytoplasmic nature. Many, however, and probably by far the largest number, are undoubtedly fragments split off from the nuclei of the leukocytes. This perhaps is shown most conclusively by the constant relation between the free bodies and partially attached nuclear fragments, and by the fact that both are produced by the shaking of blood. Why the number of such bodies should be increased in scarlet fever, measles, erysipelas, and probably other diseases remains unknown. As now understood, it does not seem possible that the bodies in question bear any causal relation to scarlet fever.

CONCLUSIONS.

Bodies in the leukocytes like those described by Döhle occur in many febrile diseases, and in smaller number in other diseases, and even in health. The larger part of these bodies are of nuclear origin, and may be produced by shaking the blood. It is not justifiable to regard them as having any etiologic significance.

OBSERVATIONS ON THE PHAGOCYTIC ACTIVITY OF THE LEUKOCYTES IN MEASLES.*

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Observations have been made on the blood of six measles patients to determine whether any changes occur in the phagocytic activity of the leukocytes during the leukopenia. Suspensions of washed blood, containing approximately the same number of polymorphonuclear neutrophiles from normal persons and from cases of measles were used. The phagocytic activity of the leukocytes was determined by comparing the number of bacteria ingested by the two sets of leukocytes under the influence of normal serum (cytophagic index). At the same time the opsonic index of the measles patients was estimated in the usual way. Typical strains of the streptococcus pyogenes, the staphylococcus aureus, and the tubercle bacillus were employed in these experiments.

In the case of all the patients examined during the leukopenia, the polymorphonuclear cells were found to be less actively phagocytic than normal cells, the average for the streptococcus being 0.53, the staphylococcus 0.6, and the tubercle bacillus 0.54, as compared with the normal standard of 1.00. In three cases examined when the number of leukocytes had become normal, the neutrophiles were found to be normally active. In one of these cases there was on the seventh day of the attack a leukocytosis, at which time the neutrophiles were more actively phagocytic. The patient had a bronchitis, which perhaps accounted for the large number of leukocytes (21,000).

The opsonic index was found to be normal except in two cases in which the streptococcus index was low. In one case examined at the end of the acute attack, the streptococcus index was slightly increased (1.4).

In order to determine whether this change in the activity of

* Received for publication September 1, 1912.

the leukocytes also occurs in experimental measles, a monkey (*Macacus rhesus*) was inoculated with measles blood and the opsonic index, and the cytophagic index to the streptococcus, the staphylococcus, and the tubercle bacillus estimated at frequent intervals. The blood was obtained from an adult woman, 24 hours after the appearance of a typical measles rash. Five cubic centimeters of blood were withdrawn from the elbow and mixed with eight cubic centimeters of citrate-salt solution (1 per cent sodium citrate in physiological salt solution). One-half of this mixture was injected intravenously and the other half intraperitoneally, about thirty minutes after the withdrawal of the blood.

At the time of the inoculation the temperature of the monkey was 104°. It gradually fell to 102.6° on the seventh day after inoculation. On the eighth day, it rose to 103.5°, falling slightly the following day, and remaining at that point during the remainder of the experiment. It is possible that the rise in the temperature on the eighth day may have been due to the measles virus.

On the seventh day the mucous membranes of the mouth appeared redder than usual, but no typical Koplik spots could be demonstrated. Twelve days after the inoculation the monkey appeared a little sick. No rash could be seen at any time.

The number of leukocytes was not increased after the injection of measles blood, but on the fifth day there commenced a distinct fall in the total number of leukocytes, lasting for 15 days. On the 22d day there was a slight leukocytosis, the number falling to normal the following day and remaining there subsequently.

The total number of neutrophiles was increased for two days following the injection, then fell below normal for 15 days, increasing again on the 21st day. The total number of lymphocytes (Chart 1) decreased on the day following the inoculation and remained low for 16 days and then returned to normal. The large mononuclear cells (Chart 1) were slightly increased on the fifth day and then remained normal during the remainder of the experiment.

The phagocytic activity of the neutrophiles (Chart 2) for the streptococcus, staphylococcus, and the tubercle bacillus was increased on the third day following the injection of measles blood.

At this time the total number of polymorphonuclear neutrophiles was increased. With the onset of the leukopenia the neutrophiles became less actively phagocytic, not returning to their normal activity until the leukopenia ceased on the 22d day. No increased activity was observed at the end of the reaction.

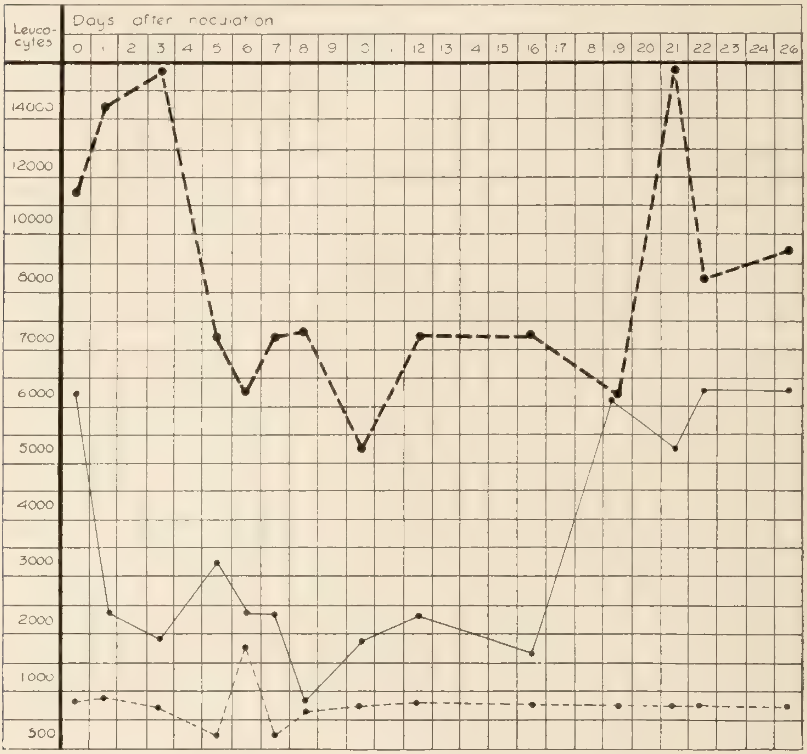


CHART 1.—Leukocyte curves in *Macacus rhesus* inoculated intravenously and intraperitoneally with 5 c.c. measles blood.

Heavy broken line = Total number of polymorphonuclear neutrophiles.
Fine broken line = Total number of large mononuclears.
Fine solid line = Total number of lymphocytes.

The opsonic index to the staphylococcus and tubercle bacillus remained about normal after the inoculation of the measles blood, but the streptococcus index rose on the third day to 1.5 and then fell below normal on the sixth day, not returning to normal until the 20th day. It is of interest to note that the patient from whom this blood was taken had a low streptococcus index throughout the

course of her infection. Just what significance this low streptococcus index might have, could not be determined.

On account of the few clinical symptoms exhibited by the monkey injected with measles blood, another monkey was inoculated with normal blood to determine whether it would produce a leukopenia and with it a decreased activity of the leukocytes.

A monkey (*Macacus rhesus*) was injected with six cubic centimeters of normal blood, mixed with eight cubic centimeters of citrate-salt solution, one-half intraperitoneally and one-half in-

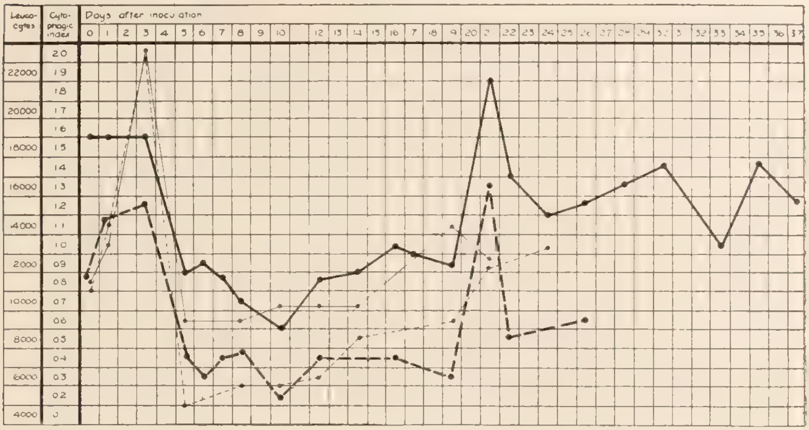


CHART 2.—Phagocytic activity of leukocytes in *Macacus rhesus* inoculated intravenously and intraperitoneally with 5 c.c. of measles blood.

Heavy solid line = Total number of leukocytes.
Heavy broken line = Total number of polymorphonuclear neutrophiles.
Fine solid line = Staphylococcus cytophagic index.
Fine broken line = Streptococcus cytophagic index.

travenously. There was no change in the temperature, and the monkey remained perfectly well. A slight increase in the number of leukocytes followed the injection, but no leukopenia occurred. The total number of neutrophiles was increased after the injection, but no greater change occurred in the number of lymphocytes and large mononuclears than occurred before the inoculation.

The phagocytic activity of the leukocytes was not affected by the injection of normal blood, the variations being within the limits of experimental error. No changes were observed in the opsonin.

Although this monkey had fewer leukocytes before the injection than the measles monkey, and the proportion of lymphocytes and neutrophiles was more nearly equal than in the measles monkey, it seems fair to assume that the leukopenia and decrease in phagocytic activity of the leukocytes produced in the monkey injected with measles blood was due to the measles virus and not altogether to the human blood.

The results of the observations on the number of leukocytes differ from those of Hektoen and Eggers and from some of those of Lucas and Prizer, in that I found no initial leukocytosis after the inoculation of measles blood.

Hektoen and Eggers[1] found that in measles in the monkey the leukocytes appear to behave very much as they do in human measles; that is, that preceded by a more or less distinct leukocytosis there occurs a leukopenia of variable degree in what would correspond in a general way to the latter part of the pre-eruptive and early part of the eruptive periods. In their animals, the leukopenia involved principally the neutrophiles, the lymphocytes being relatively somewhat increased.

Lucas and Prizer[2] observed that during the pre-eruptive stage of measles in *Macacus rhesus*, there is a leukopenia involving the polymorphonuclear neutrophiles. This leukopenia develops in from five to 10 days after the inoculation and may be preceded by a transient lymphocytic and large mononuclear leukocytosis which is probably absent or only poorly developed in the severe form of the reaction but is strongly developed in the less severe cases.

CONCLUSIONS.

From these experiments the following conclusions may be drawn:

1. The phagocytic activity of the leukocytes is decreased for the streptococcus, staphylococcus, and tubercle bacillus during the leukopenia of measles in man. Their activity becomes normal with the increase in their number.

2. The inoculation of the monkey (*Macacus rhesus*) with measles blood is followed by a leukopenia involving the polymorphonuclear

[1] *Jour. Am. Med. Assoc.*, 1911, 57, p. 1833. [2] *Jour. Med. Res.*, 1912, 26, p. 181.

neutrophiles and lymphocytes, the large mononuclear cells being slightly increased. The leukopenia is preceded and followed by an increase in the number of neutrophiles.

3. The inoculation of the monkey with measles blood produces at first an increased phagocytic activity of the leukocytes, followed by a decided decrease in their activity with the onset of the leukopenia. This decreased activity persists until the number of leukocytes becomes normal.

4. The injection of normal blood into the monkey produces a slight leukocytosis, but no leukopenia and no change in the phagocytic activity of the leukocytes. The total number of neutrophiles is slightly increased, but no marked changes are observed in the lymphocytes and large mononuclear cells.

Finally I would suggest that the leukopenia and the diminution of the phagocytic activity of the leukocytes in measles may account in some degree for the frequency of secondary infections in this disease.

THE ACTION ON DOGS OF THE TOXIC SUBSTANCE OBTAINABLE FROM VIRULENT PNEUMOCOCCI AND PNEUMONIC LUNGS.*

E. C. Rosenow and Aaron Arkin.

(*From the Memorial Institute for Infectious Diseases, Chicago.*)

From the work of Gay and Auer and Lewis,[1] Schultz and Jordan[2] and others we know that death in guinea-pigs in immediate anaphylaxis is due to bronchial spasm, and there is no doubt that death is due to the same cause after injections of peptone, of "anaphylatoxin," and of the toxic material obtainable on autolysis in NaCl solution of various bacteria.

Biedl and Kraus,[3] Pearce and Eisenbrey,[4] and others have shown that the marked fall in blood-pressure is the important symptom in anaphylaxis in dogs and that the other symptoms, wretching, vomiting, defecation, urination, and prostration, are secondary to anemia of the brain. Schittenhelm and Weichardt[5] have called particular attention to the lesions found in the intestines in anaphylaxis in dogs, and name the condition "enteritis anaphylactica."

In previous studies one of us[6] has shown that autolytic extracts of various bacteria, especially pneumococci, produce in normal guinea-pigs symptoms typical of immediate anaphylaxis. The extracts are, relatively speaking, nontoxic at first, very toxic at a certain period, and become nontoxic after being kept at 37° C. This condition is associated with proteolysis. It has been shown further that the symptoms in guinea-pigs caused by autolytic extracts of pneumococci in no way differ from those observed following the injection of toxic material from pneumococcus exudates. If it could be shown that the symptoms in dogs following the injection of toxic extracts are more or less typical of

* Received for publication September 1, 1912.

[1] *Jour. Exper. Med.*, 1910, 12, p. 151.

[2] *Jour. Pharm. and Exper. Therap.*, 1911, 2, p. 375.

[3] *Wien. klin. Wchnschr.*, 1909, 22, p. 363.

[4] *Jour. Infect. Dis.*, 1910, 7, p. 565.

[5] *Deutsch. med. Wchnschr.*, 1911, 37, p. 867.

[6] Rosenow, *Jour. Infect. Dis.*, 1911, 9, p. 190. 1912, 11, p. 235.

anaphylaxis in this species, it would furnish additional evidence that during anaphylaxis there is produced in the animal toxic material similar to that produced *in vitro*.

In the following pages we wish to record briefly the results of experiments in dogs with intravenous injections of various products of pneumococci and pneumococcus exudates.

TECHNIC.

Ether was used as the anesthetic in all of the experiments in which tracings of the blood-pressure and respiration were made. A glass cannula was inserted into the trachea through a median incision and connected with the ether bottle by means of a T-tube. To the small arm of this tube was connected the respiratory tambour. A three-way cannula was placed in the carotid artery after ligitation of the vessel distally. One arm of this cannula was connected with a pressure-bottle containing 2 per cent sodium citrate solution. The other arm was connected with the mercury manometer for recording the blood-pressure. The injections were all made into the femoral vein by exposing the vein. Before the injection, complete anesthesia was induced and kept up throughout the experiment. The supply of ether was kept constant by adding sufficient at intervals to keep the level of the liquid the same throughout the experiment. After the completion of the experiment the dog was immediately killed, provided it did not die as a result of the injection, and autopsy made. Sections of the organs were immediately placed in Zenker's fluid. The sections were stained mostly with hematoxylin and eosin, and many sections studied from each organ.

The extracts of pneumococci were prepared from virulent pneumococci which had been recently isolated and cultivated in ascites-meat broth. They were sedimented at high speed and the supernatant broth poured off. The density of the suspensions in NaCl solution was between four and five times that of a dense growth in the broth. For autolysis the pneumococci were suspended for 48 hours in each of two changes of NaCl solution at 37° C., and practically all became gram negative. In the NaCl suspension of living, unautolyzed pneumococci injected in Experiment 3

approximately one-sixth of the cocci had become gram negative at the time of the injection. The extract which was injected into the sensitized dog (Experiment 5), was prepared by placing the suspension of pneumococci in the ice-chest for 48 hours and heating to 60° C. for one hour. It was then centrifugated clear. The extracts of pneumonic lungs were prepared by passing a portion of a freshly obtained lung through a meat chopper, mixing with NaCl solution, and straining through a rather thick layer of sterile cheesecloth. This was then centrifugated at high speed, and the supernatant opalescent fluid used. This is designated as the first extract, while the extract of the sediment thus obtained is designated as the second extract. Only such lungs were used as contained pneumococci in apparently pure culture. Control cultures were made to determine whether the extract was sterile; only sterile extracts were used. The doses for dogs, per gram of body weight, were approximately four-fifths of the average dose for guinea-pigs. It was found necessary to give rather large doses in order to produce striking results. The temperature was taken at intervals throughout the experiment but showed little change. A drop of one-half to one degree Centigrade was noted in the fatal cases. In one of the dogs, which showed marked immediate symptoms, the leukocyte count presented a pronounced drop. The coagulation time of the blood was noted at the end of the experiments. The 10 experiments selected for publication illustrate the results obtained in a larger series. It was noted that gaseous distension and acute dilatation of the stomach were present in those which showed marked symptoms. When this fact was mentioned to Dr. R. T. Woodyatt, he suggested that we test the gas for CO_2. This was done by passing it through lime water. Control tests for fermentation of the stomach contents were placed in the incubator, but in no instance was fermentation observed.

RESULTS.

The results in Experiments 1 and 3 need a word of explanation. The early effect on the blood-pressure in Experiment 1, in which pneumococci partially autolyzed under ether were injected and which contained a large amount of preformed toxic material, is

greater than in Experiment 2, in which live virulent pneumococci were injected. The transient early drop observed in No. 3 is undoubtedly due to free toxic material because some of the pneumococci were gram negative; the hemorrhages and the late effect on the blood-pressure in this experiment are marked. This is what one would expect because unautolyzed live pneumococci undergo destruction *in vivo* in large numbers and hence liberate a proportionately greater amount of toxic material. It is possible too that the late toxic effects were due to bacterial growth. The fact that the more completely autolyzed extract in Experiment 2 and the autolyzed pneumococci in Experiment 4 produced no symptoms, as well as the results of other experiments, speak for the correctness of the view that the toxic material, in the main, is similar whether produced by self-digestion of pneumococci *in vitro* or by lysis *in vivo*.

In studying the protocols and curves it is apparent that the evidence of splanchnic dilatation and intestinal lesions is most marked in the normal animals which showed immediate and pronounced fall in blood-pressure as the result of injection of preformed toxic material, and in sensitized animals following injection of unautolyzed extracts or suspensions of pneumococci. In those injected with the second unautolyzed lung extracts (Experiments 7 and 8), in which the fall in blood-pressure is more gradual and begins one hour after the injection, the intestinal lesions are less pronounced. By injecting suspensions of pneumococci into unetherized, sensitized dogs, and toxic autolysates and toxic extracts from pneumonic lungs in normal dogs, the typical symptoms and findings described by various observers in protein anaphylaxis were obtained in each case. The fact that typical intestinal lesions, the "enteritis anaphylactica" of Schittenhelm and Weichardt, can be produced by preformed toxic material in normal dogs indicates that in anaphylaxis this lesion is due at least in part to marked splanchnic dilatation and not wholly to the formation of toxic material in the intestinal wall, as these authors conclude. The reason that the lesions in the sensitized dog (Experiment 5) are not more pronounced is due to the fact that the extract used contained only a small amount of material from which toxic matter could be

formed. Control injections into unetherized, sensitized dogs of corresponding suspensions of pneumococci produced extremely severe symptoms. A normal dog injected with this unautolyzed heated extract showed no noteworthy symptoms.

The results in Experiments 7, 8, and 10 are interesting. They show that the second extract of pneumonic lungs is not immediately toxic, and not toxic when kept on ice for 24 hours. Both, however, contained material from which toxic products are formed *in vivo*. After this extract was allowed to autolyze under ether for five days (Experiment 10) no immediate symptoms were produced, and the material from which toxic matter was produced previously *in vivo* had then disappeared. Injections of lung extract 649 showed that it was very toxic at once and only slightly toxic at the end of 18 hours when kept at 37° C.; whereas, when new pneumococci were added (Experiment 9), it was highly toxic at the end of 18 hours. Other experiments gave similar results. We realize that extracts of normal lungs and other organs produce similar symptoms, but the fact that addition of pneumococci to these extracts increases the toxicity, and that the toxicity appears and disappears in the same way as in pneumococcus autolysates, makes it clear that the pneumococci play a part.

The hemorrhages were most extreme in the dogs which received the large doses of living virulent pneumococci, the first extract of the pneumonic lungs, and a lung extract to which virulent pneumococci were added and then allowed to autolyze under ether at 37° C. for 18 hours. The dogs which gave no symptoms showed few or no hemorrhages. The perivascular distribution of the hemorrhages in the lung and other organs is particularly interesting. The exact reason for this is not entirely clear, but concentration of toxic material in the vessels—a form of mass action—is probably responsible. The order of frequency in which hemorrhages were found in the viscera is as follows: lungs, heart, kidney, intestines, liver, pancreas, and spleen.

The coagulation time of the blood was markedly delayed, for hours, in the dogs which died as the result of the injections; moderately delayed in the dogs which showed severe symptoms; and unchanged in those which showed no symptoms.

Gaseous distension of stomach, amounting to acute dilatation in Experiments 7 and 9, was present to a greater or less degree in all the animals which showed symptoms. No gas was found in those which showed no symptoms. Carbon dioxid was found present in large amounts in the stomachs which contained gas. The result in one normal dog (unanesthetized), which was injected with a large amount of toxic material from a pneumonic lung, and in two sensitized dogs, which received a very large dose of living virulent pneumococci, should be mentioned in this connection. They all died within 24 hours with a most extensive intestinal hemorrhage, bloody diarrhea, etc., the exact picture of "enteritis anaphylactica" described by Schittenhelm and Weichardt. The stomach contained a moderate amount, the intestine a large amount, of bloody, frothy fluid. Carbon dioxid was demonstrated in both cases, and control tests of the respective materials showed no fermentation. The significance of the presence of carbon dioxid in the stomach and intestine has recently been discussed quite fully by Woodyatt and Graham.[1] Suffice it to say that it is difficult to understand how such a large quantity could accumulate except it be excreted into stomach and bowel from the circulating blood. It should be stated that one of us has found CO_2 in the stomach quite frequently in guinea-pigs which die in from six to 24 hours after injection of toxic pneumococcus extracts and from acute pneumococcemia, and that there is present a great tendency to postmortem digestion of the stomach in these animals.

A study of the curves shows that a second toxic dose produces markedly less disturbance than the first dose. In the dogs which received first a nontoxic injection and then a highly toxic dose this insusceptibility is not observed. Renal secretion is practically suspended in the susceptible animal and active in the insusceptible. It is likely that the insusceptibility is explainable on the same basis as antianaphylaxis in general.

Control injections in normal guinea-pigs showed that the appearance and disappearance of toxicity occurs simultaneously for the two species, the striking symptom in the guinea-pig being bronchial spasm while the drop in blood-pressure is the striking

[1] *Trans. Chicago Path. Soc.*, 1912, 8, p. 353.

symptom in the dog, though hemorrhages are more marked in the latter species.

The effect on the respiration is not as marked nor as constant as on blood-pressure. Almost immediately after the injection of toxic material in normal dogs, and of nontoxic heated extract in sensitized dogs which recover, there is a transient increase in amplitude and rate, followed, in a few moments, by a greatly diminished amplitude associated with an inspiratory position of the lungs. This, in turn, is followed by a markedly increased amplitude as the blood-pressure rises. In the animals which succumb, the respiration, when the toxic symptoms begin, is depressed and finally paralyzed. The respiration usually ceases before the heart stops. The increased respiration in dogs which recover is not wholly dependent on return of blood-pressure because the amplitude is greater than before the injection, the blood-pressure still being depressed.

The symptoms and the postmortem changes in dogs are strikingly like those of anaphylaxis. Biedl and Kraus and Pearce and Eisenbrey have shown that in anaphylaxis in the dog the action is peripheral, and Rosenow has shown that the action of the toxic substances in question is also peripheral in normal guinea-pigs. Hence it is likely that the action in dogs following injections of our toxic material is mainly peripheral. The marked cyanosis, the extensive hemorrhages, the delayed coagulation of the blood, and the presence of carbon dioxid in the stomach of the animals which show marked symptoms, speak strongly in favor of the view that, whatever the primary effect of the toxic substances may be, one of the chief results following their injection is interference with oxidation processes. Therefore the increased respiration observed rather late in the animals which recover may well be due to stimulation of the respiratory center by carbon dioxid which accumulates because of the interference with oxidation. In the same way it would seem that, in the animals which succumb, the respiratory center is overstimulated and finally paralyzed. The early and transient inhibitory effect on the respiration would also speak in favor of this view. Detoxication of the material injected

occurs and recovery ensues when the blood becomes thoroughly aerated. This does not occur in the animals which die, cyanosis becoming most extreme.

The pneumonic lung extracts, before they have become acutely toxic and after the toxicity (both for dogs and guinea-pigs) has disappeared, show little or no increase in amino-nitrogen. After long residence at 37° C., however, they show a decided increase and it therefore seems that a certain amount of protein splitting takes place early even though it is not measurable by formol titration. In this respect the lung extracts behave as do pneumococcus extracts and serum mixtures. The toxicity appears and disappears while formol titration shows little or no protein cleavage. Polariscopic measurements, however, show a diminished rotatory power. Except in unimportant particulars the general character of the curves and the lesions produced is strikingly similar, whether the toxic material comes from pneumococci only or from pneumonic lungs and whether formed *in vivo* or *in vitro*. The difference in degree of lesions is due, we believe, rather to a difference in the total dose and concentration at any one time than to a difference in the character of the toxic substances formed. It is of course likely that the lung extracts contain toxic substances which are not present in the extracts of pneumococci. The chief toxic substance, however, is probably identical because of the similarity of symptoms produced and because it appears and disappears under the same conditions in each case. Extracts of pneumococci or of consolidated lungs, which are immediately toxic, are potentially less toxic than those in which free toxic material has not yet been formed, but which is elaborated in the animal after injection. Here all the toxic matter exerts its toxic effects as it is made, whereas during the formation of the toxic material *in vitro* some undoubtedly has gone by the toxic stage at the time of injection and the supply of the substance from which the toxic matter is made is proportionately reduced. Hence the tendency of the animal to recover, provided the initial dose does not kill it outright. That this interpretation is correct seems likely, because after self-digestion continues for a longer time both the immediate as well as the late toxic effects are no longer observed.

CONCLUSIONS.

The action of the toxic substances obtainable from pneumococci and pneumococcus exudates on the blood-pressure and respiration in normal dogs is identical and strikingly like that which is observed in immediate anaphylaxis in dogs. Pneumococcus anaphylaxis in dogs does not essentially differ from protein anaphylaxis in general. The lesions are also similar. The appearance and disappearance of the toxic substance or substances in pneumococcus autolysates and pneumonic lung extracts occurs under the same conditions and simultaneously for dogs and guinea-pigs. In the former species, the striking effect is the marked drop in blood-pressure; in the latter it is the bronchial spasm. Hemorrhages in general, and especially intestinal hemorrhages, are more pronounced in dogs than in guinea-pigs.

The hemorrhages, the effect on the respiration, the extreme grade of cyanosis, especially in the fatal cases, the delayed coagulation of the blood, and the presence of carbon dioxid in the stomach indicate that one of the chief effects of the toxic substances is an interference with the normal oxidative processes. The toxic substances concerned are probably of the same general nature because the lesions and the effect on the circulation and respiration are so similar, and it makes no essential difference whether they are formed *in vitro*, in the consolidated lung in man, at once in sensitized dogs, or at a later period in normal dogs.

EXPERIMENT 1.

EFFECT OF THE INJECTION OF TOXIC PNEUMOCOCCUS EXTRACT (NO. 300) ON THE BLOOD-PRESSURE AND RESPIRATION.

Female dog, weight 8 kilos. Ether anesthesia.

Time		Injection	Blood-Pressure	Respiration	
				Rate	Amplitude
Hrs.	Min.				
12	25	Injected about 15 c.c.	144 mm.	45	14 mm.
12	27		64	50	19
12	30	Injected 6 oz. 12:29 to 12:31	126	48	....
12	32		44	42	8
12	37		44	54	20
12	45	Injected 7 oz. 12:45 to 12:47	60	63	25
12	48		82	75	30
12	52		112	70	29
12	58		128	60	25
1	10½		132	55	25

Dog was killed at 1:20 P.M.

Autopsy.—The heart is dilated with blood. The musculature is quite soft. There are no visible hemorrhages in the endocardium. The lungs present several areas of hemorrhage, especially in the dorsal portion of the lobes. These are about one centimeter in diameter. The lungs float on the surface of water. There is a marked congestion of the right posterior lobe.

The stomach is greatly distended with gas which gives the test for carbon dioxid (lime-water test). There are no hemorrhages. The spleen is somewhat darker than normal. The kidneys are somewhat enlarged; the cortex is especially thickened and rather cloudy; the cortical striations are rather indistinct. The capsule strips readily. There seems to be a marked congestion of both kidneys. The liver, pancreas, etc., appear unchanged.

Microscopical changes.—There is a moderate grade of hyperemia of the villi in the intestine. The kidney presents marked changes; many glomeruli contain blood within the Bowman's capsule; some of the glomerular tufts are distended with blood; in other areas there are circumscribed hemorrhages which seem to occupy the spaces of Bowman's capsule. There are other hemorrhages in the cortex and few between the tubules. In the lung is found a very striking condition of hemorrhages, surrounding the larger vessels, especially veins. Some of these are so extensive as to surround the vessel completely, forming a ring of blood in the loose connective tissue. There are hemorrhages into the alveoli in many areas. The spleen shows a marked congestion. In the heart muscle can be found a few small hemorrhages between the muscle fibers. The other organs present no changes.

EXPERIMENT 2.

Effect of the Injection of Autolyzed Pneumococcus Extract (No. 301) after Toxicity for Guinea-Pigs Had Disappeared.
Female dog, weight 11 kilos. Ether anesthesia.

Time	Injection	Blood-Pressure	Respiration	
			Rate	Amplitude
Hrs. Min.				
10 26	Injection of 5 oz. from 10:26 to 10:27½	144 mm.	66	10 mm.
10 27		132	..	9.
10 27¼		139	..	9.
10 28		132	74	10.
10 30		151	66	10.
10 32		154	64	11 5
10 40		158	63	12 (av.)
11 05		161	58	11 5 (av.)

Autopsy.—Since a second injection of a toxic extract was made in this animal not shown in the curve, and which caused a marked fall of blood-pressure, the changes in the organs were not carefully studied.

EXPERIMENT 3.

EFFECT OF THE INJECTION OF UNAUTOLIZED LIVING VIRULENT PNEUMOCOCCI ON THE BLOOD-PRESSURE AND RESPIRATION.

Male dog, weight 9 kilos. Ether anesthesia.

Time		Injection	Blood-Pressure	Respiration	
				Rate	Amplitude
Hrs.	Min.				
1	04	Injection of 1 oz. of suspension (containing 700 billions in salt solution.) Time of injection 20 seconds.	152 mm.	52	11. mm.
1	05		54	86	14. (Expiration forcible.)
1	08		70	60	12.
1	10		92	58	12.
1	13		100	62	14.
1	17		106	64	19.
2	00		124	62	32.
2	30		116	56	14.
3	22		82	52	27.
3	30		78	52	30.
3	40		76	54	28.
5	46		60	40	13.
5	49		59	35	10.
5	55		59	32	11.5
5	56		59	..	11.

Autopsy.—The heart is greatly dilated, soft, somewhat flabby. There are subepicardial hemorrhages, from a pin-point to 0.5 cm. in diameter, over the left ventricle, especially near the apex. The inner wall of the left ventricle is covered with subendocardial hemorrhages, varying from one or two millimeters to one centimeter in diameter. These are most marked over the papillary muscles, one of which is capped by a large, deep hemorrhage. Some hemorrhages extend to the attachments of the mitral valve; they are very numerous on the septal wall of the left ventricle. There are no visible subendocardial hemorrhages in the right heart. The right posterior lobe of the lung is very dark red and sinks in water; there is a thick bloody mucus in the bronchi; the rest of the lung is collapsed. Other lobes are redder than normal and less crepitant. The peribronchial glands are enlarged. The liver is dark in color, but there are no other visible changes. The stomach is distended with gas, and contains food; CO_2 test positive; the veins are especially enlarged. The kidneys contain a large amount of blood; the cortical markings are distinct. In the intestines there is present a small amount of bloody mucous fluid. The lymph follicles are large and hyperemic. There are small areas of mucosa which are red in color and there appear to be hemorrhages.

Microscopical changes.—Changes are most numerous in the heart. There are hemorrhages in the papillary muscles, which are most often near the junction of the muscle fibers with the fibrous tissue, between the fibers, and which have a tendency to run parallel with the muscle fibers, communicating with each other. Some of the hemorrhages are just beneath the endocardium. In the lung are numerous hemorrhages; many of the alveoli and bronchioles are filled with blood. There are perivascular hemorrhages in the liver; these are chiefly about the portal veins in the portal canals. Some of the veins are surrounded by blood which has dissected the loose connective tissue. In the villi of the intestine there is marked hyperemia so that the capillaries appear to be on the surface of the villi. The ends of the villi are chiefly

affected, and here one can see the ring of dilated capillaries at the circumference of the villus with the large central capillary, but no hemorrhage is present. In the cortex of the kidney are numerous hemorrhages, mostly about three times the size of a Malpighian body. Some of the glomeruli are filled with blood. There are some hemorrhages between the tubules. There are small hemorrhages in the cortex of the adrenal and the medulla is congested.

EXPERIMENT 4.

EFFECT OF THE INJECTION OF AUTOLYZED VIRULENT PNEUMOCOCCI ON THE BLOOD-PRESSURE AND RESPIRATION.

Female dog, weight 8 kilos. Ether anesthesia.

Time		Injection	Blood-Pressure	Respiration	
				Rate	Amplitude
Hrs.	Min.				
2	39	Injection of 6 oz. (containing 700 billions in salt sol.) from 2:39 to 2:40½	140 mm.	86	13. mm.
2	40		137	...	9.
2	40½		142	106	9.
2	45		135	90	12.5
2	56		140	96	10.
3	10		140	100	11.
3	40		134	88	10.5
3	42		133	...	10.5

Dog was killed at 4:00 P.M.

Autopsy.—There are no marked changes in the lungs. Two or three small areas of hemorrhage are present on the surface, about five millimeters in diameter. There are a few petechiae over the region of the interventricular septum. The abdominal organs are unchanged. The stomach contains no gas. Microscopically no changes are present in any of the organs, except for a few hemorrhages in the lungs and heart muscle.

EXPERIMENT 5.

EFFECT OF THE INJECTION OF HEATED UNAUTOLYZED PNEUMOCOCCUS EXTRACT (NO. 302) ON THE BLOOD-PRESSURE AND RESPIRATION OF SENSITIZED DOG.

Male dog, weight 7 kilos. Ether anesthesia.

Time		Injection	Blood-Pressure	Respiration	
				Rate	Amplitude
Hrs.	Min.				
2	55	Injection of 5½ oz. of extract from 2:55 to 2:56½	98 mm.	50	5. mm.
2	55½		105	50	5.
2	55¾		40	30	1.5
2	56½		65	50	4.
3	00		78	40	3.
3	06		56	40	3.
3	11		44	38	4.
3	24		65	50	5.5
3	27½		76	60	6.5
3	50		73	36	5.5
3	55		70	34	5.

Dog was killed at 4:00 P.M.

Autopsy.—There are several areas of hemorrhage in the lungs, some as large as two centimeters in diameter. There is some edema of the lungs. The heart is dilated and quite soft. The liver is dark in color, with marked congestion. The stomach is distended with gas, carbon dioxid test positive. There are no visible changes in the bowels except a moderate congestion. The cortex of the kidney is pale, but distinct.

EXPERIMENT 6.

EFFECT OF THE INJECTION OF EXTRACT (NO. 637) OF PNEUMONIC LUNG (IN SALT SOLUTION) ON THE BLOOD-PRESSURE AND RESPIRATION.

Female dog, weight 7 kilos. Ether anesthesia.

Time		Injection	Blood-Pressure	Respiration	
				Rate	Amplitude
Hrs.	Min.				
2	30	Injection of 4 oz. of extract from 2:30 to 2:32½	136 mm.	55	12. mm.
2	30⅓		160	..	12.
2	30½		72	..	10.
2	30⅔		102	..	160.
2	30⅚		66	..	....
2	31⅓		78	46	90.
2	32		52	62	10.
2	33		48	55	0 4
2	36		42	65	12.
2	39		50	55	13.
2	45		64	58	50.
2	48	Injection of 4 oz. of extract from 2:48 to 2:50	78	60	50.
2	48⅓		92	..	40.
2	48½		52	..	40.
2	50		103	80	80.
3	00		103	70	40.

Autopsy.—The heart is filled with a large amount of blood. There are several areas of subendocardial hemorrhages of from one to five millimeters in diameter, scattered chiefly through the right ventricle, especially about the papillary muscle; there are few hemorrhages in the left ventricle. The lungs are markedly congested, especially in the dorsal portions. A few areas of probable hemorrhage can be seen on the surface. The stomach is large and distended with gas. There are many irregular hemorrhages under the serosa; these are light red in color and resemble exudations. The splanchnic vessels are greatly distended, especially the mesenteric veins. Some bloody fluid is present in portions of the mesentery, near its attachment to the bowel. On incision, the small bowel is found to be filled with a bloody, frothy fluid. The mucosa is covered with a sticky mucus which almost forms a membrane. On clearing the surface many large elevated blood-stained regions are found scattered through the bowel, varying in size from one-half to two centimeters in diameter. Some are ovoid, with their long diameter longitudinal. There are also a few small defects in the mucous membrane. The mucosa is covered in large areas with small and large submucosal hemorrhages, which are present through almost the entire bowel. The pancreas is studded with petechial hemorrhages. The spleen is dark in color. The liver is dark, and a large amount of blood exudes from the cut surfaces. The kidneys are congested, especially the cortex near the medulla. The adrenal medulla is very dark in color.

Microscopical changes.—In the lymphoid follicles of the intestine are numerous hemorrhages, some so large as to occupy almost the entire follicle. The centers of all the follicles are filled with blood. There are also submucosal hemorrhages, and hemorrhages between the villi. There are large hemorrhages in the mesenteric lymph glands. In the lungs are large hemorrhages about some of the larger vessels. The spleen is markedly congested and hemorrhagic. The hemorrhages are more numerous about the vessels. There are minute hemorrhages scattered through the heart muscle, between the fibers; there are also a few large hemorrhages. There are many small distinct hemorrhages in the adrenal cortex, and the medulla is congested. A few irregular regions of hemorrhage are found in the liver. In the kidney are numerous hemorrhages in the cortex, many into the glomeruli. These are mostly large, round or oval, circumscribed regions. There are hemorrhages in the medulla between the tubules. Some of the tubules contain blood.

EXPERIMENT 7.

Effect of the Injection of Second Extract of Pneumonic Lung (No. 639), as Soon as Made, on the Blood-Pressure and Respiration.

Female dog, weight 10 kilos. Ether anesthesia.

Time		Injection	Blood-Pressure	Respiration	
				Rate	Amplitude
Hrs.	Min.				
3	00	Injection of 5½ oz. of extract from 3:00 to 3:02½	148 mm.	48	15. mm.
3	02½		142	44	17.
3	07		160	39	15.5
3	11	Injection repeated	162	38	16.
3	13½		148	46	15.
3	17		158	40	13.
3	30		168	30	11.
3	45		179	20	6.
3	51		156	14	7.5
3	56		132	10	11.
4	00		94	6	10.
4	02	Heart stopped	10	..	....
4	04½	Respiration ceased	......	..	....

Autopsy.—The heart is greatly dilated and very dark in color; the coronary veins are very distinct and dark in color. The blood is almost blue. The lungs are collapsed. There is passive hyperemia of the dorsal borders. The stomach is large and distended with gas, CO_2 test positive. The mesenteric veins are greatly distended, dark blue and distinct. The liver is extremely dark, bluish-purple in color, and contains a large amount of blood. The pancreas and spleen appear normal. The veins of the renal capsule are very distinct; the capsule strips readily, disclosing small circumscribed dark regions in the cortex. There are no other changes.

Microscopical changes.—There are small hemorrhages in the lung, chiefly beneath the pleura. The bronchial glands are normal. There are large perivascular hemorrhages in the pancreas, especially about the larger vessels, also minute hemorrhages between the gland-cells and into the connective tissue. The kidney presents numerous hemorrhages, chiefly in the cortex; in some regions the glomeruli are filled with blood and even distended, so that the glomerular tuft is not seen. The liver shows a high grade of hyperemia, the congestion being chiefly in the centers of the lobules. The capillaries and veins of the heart muscle are greatly distended.

EXPERIMENT 8.

EFFECT OF THE INJECTION OF THE SECOND EXTRACT OF PNEUMONIC LUNG (NO. 639), AFTER ON ICE FOR 24 HOURS, ON THE BLOOD-PRESSURE AND RESPIRATION.
Female dog, weight 5.2 kilos. Ether anesthesia.

TIME		INJECTION	BLOOD-PRESSURE	RESPIRATION	
				Rate	Amplitude
Hrs.	Min.				
12	16	Injection of 2½ oz. of extract from 12:16 to 12:17	113 mm.	95	7. mm.
12	16¼		117	90	6.5
12	16½		106	85	6.5
12	17		110	95	7.
12	22		111	90	6.
12	27		103	98	4.5
12	30	Injection of 3½ oz. of extract from 12:30 to 12:34	110	88	5.
12	34		112	80	5.
12	40		126	70	4.
12	45		127	56	4.
12	49		124	52	3.8
1	00		130	44	2.8
1	05		124	36	2.
1	08		114	40	1.8
1	11		93	36	1.5
1	14½		49	30	1.5
1	15		41	15	2.
1	16	Respiration ceased	....	..	....
1	17	Heart stopped	0	..	....

Autopsy.—There are no lesions, except for the dilated heart and congestion of the dorsal portion of the lung.

Microscopical changes.—There are frequent hemorrhages in the renal cortex; some seem to be in Bowman's capsule. The capillaries and veins of the heart muscle are dilated.

EXPERIMENT 9.

EFFECT OF THE INJECTION OF LUNG EXTRACT (NO. 649) AFTER 18 HOURS AT 37° C. PLUS GROWTH FROM 300 C.C. BROTH CULTURE OF PNEUMOCOCCUS ON THE BLOOD-PRESSURE AND RESPIRATION.
Male dog, weight 8 kilos. Ether anesthesia.

TIME		INJECTION	BLOOD-PRESSURE	RESPIRATION	
				Rate	Amplitude
Hrs.	Min.				
12	24	Injection of 6 oz. of extract from 12:24 to 12:25	100 mm.	50	9. mm.
12	24⅙		107	..	....
12	24⅚		47	62	11.
12	25½		68	72	12.
12	27½		87	66	8.
12	31		41	62	11.
12	36		34	70	13.
12	44		42	68	15.
1	38		32	45	10.5
2	05		29	32	5.
2	37		22	8	6.
2	48	Respiration ceased	....	..	....
2	54	Heart stopped	....	..	....

Autopsy.—The lungs are collapsed, of a somewhat leathery consistency, and dark red in color, especially on the dorsal surface. The cut section has a leathery appearance and is quite bloody. The heart is markedly dilated, and soft. The intestines on cross-section show what appears to be submucous hemorrhage involving the entire circumference of the bowel. There is a large amount of frothy fluid in the intestine, which is partly bloody. The stomach is distended with gas and a frothy greenish fluid. The other organs present no gross changes.

Microscopical changes.—The villi of the intestine present an extreme capillary dilatation, so that the capillaries lie on the surface of the villi. These form a ring beneath the surface almost in contact with each other, with one dilated central capillary, whereas in the normal intestine capillaries are found with difficulty. The entire circumference of the bowel presents this beautiful picture of congestion. There are also many hemorrhages at the ends of the villi and in the submucosa. In the pancreas there is a congestion of the capillaries and veins. There are a few small hemorrhages. In the lung the hemorrhages are most marked. Many regions contain alveoli filled with blood. Some of the bronchioles also contain blood. In the liver are a few small hemorrhages between the liver cells, with some congestion. The spleen is markedly congested and contains hemorrhages. In the heart muscle are congested capillaries and venules with a few small hemorrhages between the muscle fibers.

EXPERIMENT 10.

EFFECT OF THE INJECTION OF AUTOLYZED EXTRACT OF PNEUMONIC LUNG (NO. 639), AFTER FIVE DAYS AT 37° C., ON THE BLOOD-PRESSURE AND RESPIRATION.

Female dog, weight 8 kilos. Ether anesthesia.

Time		Injection	Blood-Pressure	Respiration	
				Rate	Amplitude
Hrs.	Min				
12	30	Injection of 5 oz. of extract from 12:30 to 12:32½	119 mm.	48	10 mm
12	32½		112	50	12
12	34		122	56	10
12	37		124	56	10 5
12	41		125	54	10
12	42	Injection of 5 oz. of extract from 12:42 to 12:44	.		
12	44		110	60	7
12	52		124	60	10
1	02		126	56	10 5
1	15		128	56	10 5
1	31		127	60	11
1	42		128	60	10.
2	00	Dog killed	128		....

Autopsy.—There are no macroscopic or microscopic changes visible in any of the organs.

EXPLANATION OF PLATES 4–14.

Curves of Respiration in Dogs 1–10.

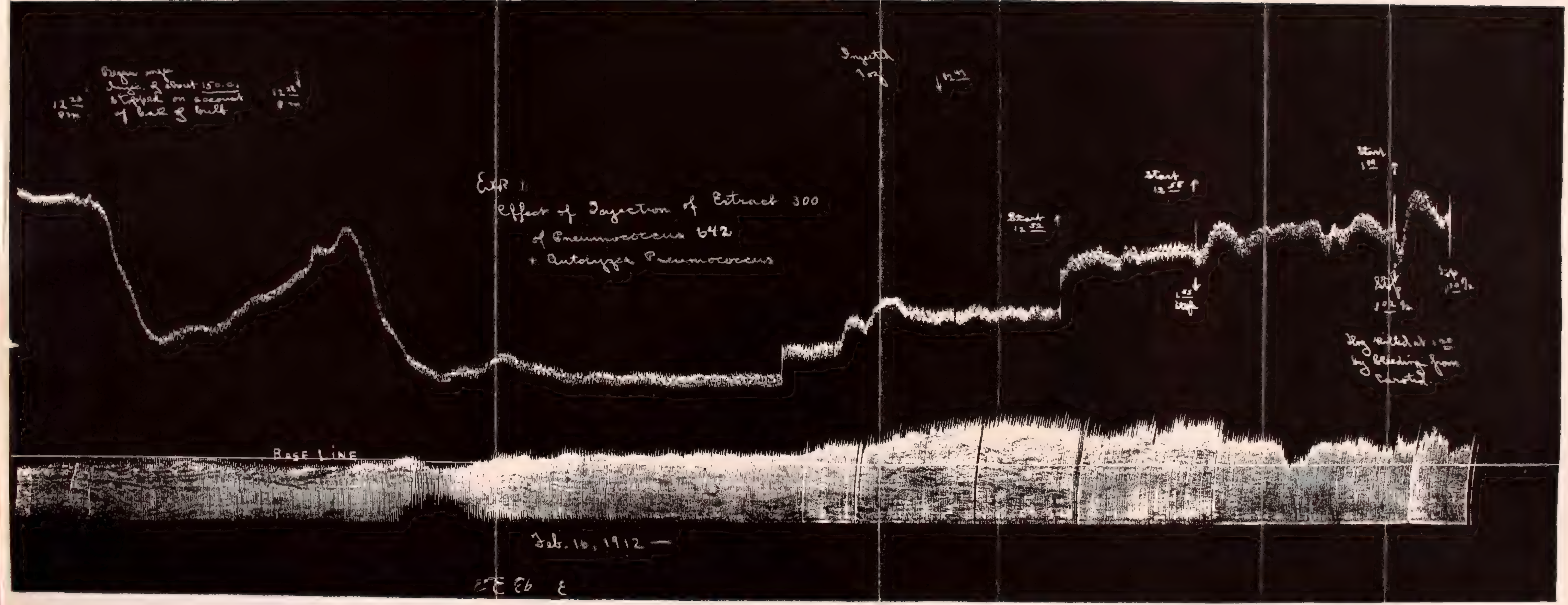
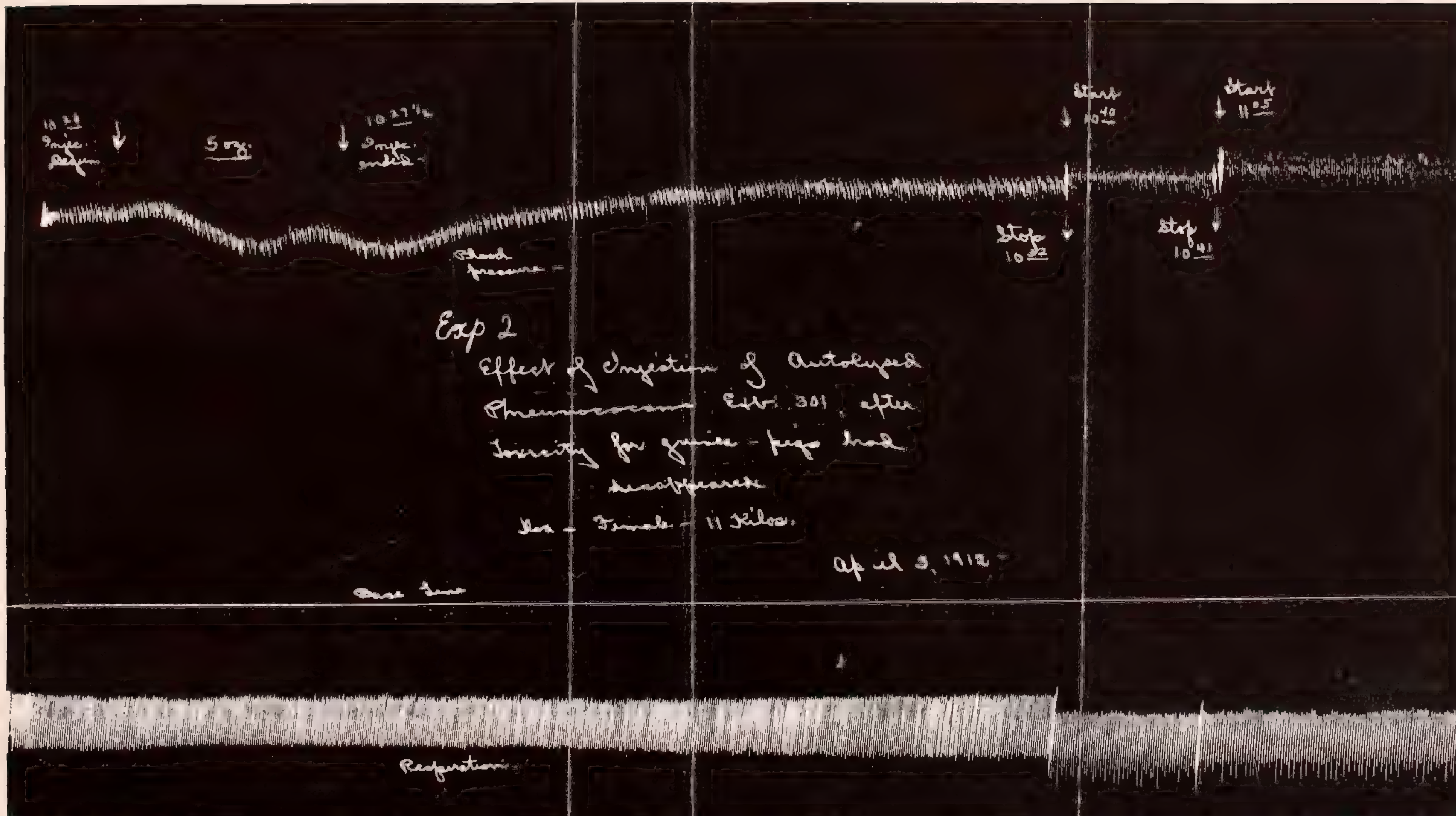

Effect of Injection of Extract 300
of Pneumococcus 642
+ Autolyzed Pneumococcus
Base Line
Feb. 16, 1912
Start
Stop
Dog killed at 1 by bleeding from carotid.

PLATE 5.


Start
Start
5 oz.
Stop
Stop
Blood pressure
Exp 2
Effect of Injection of Autolysed
Base line
Respiration

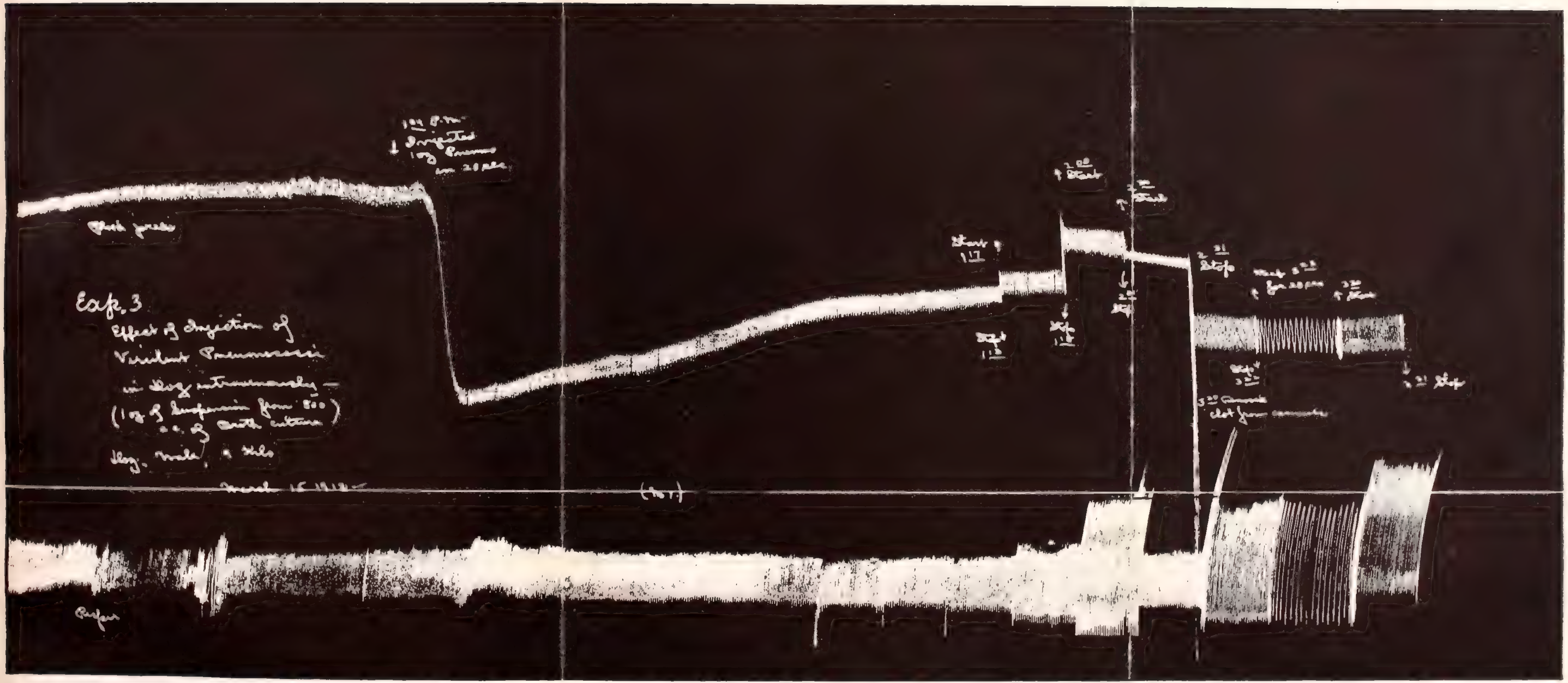
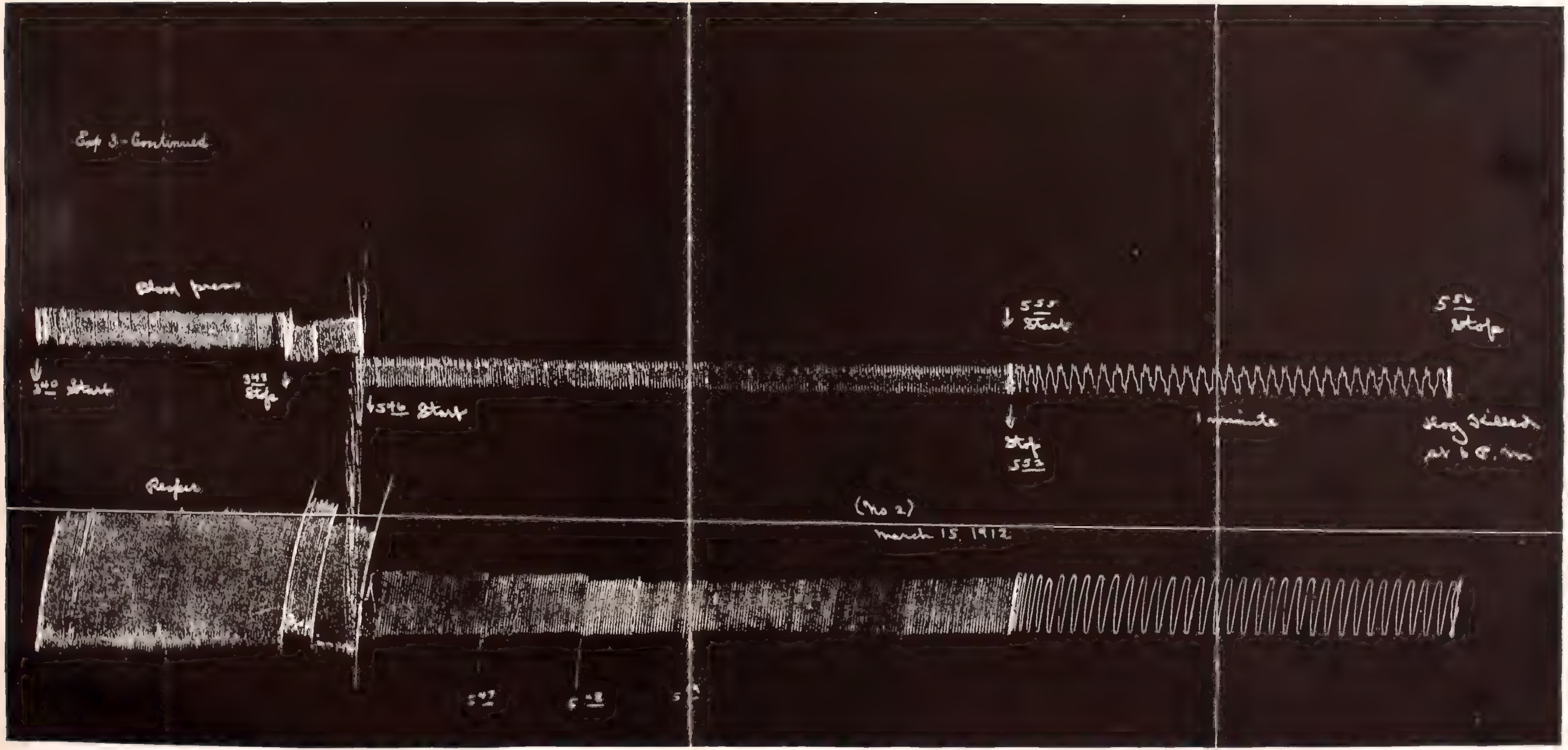
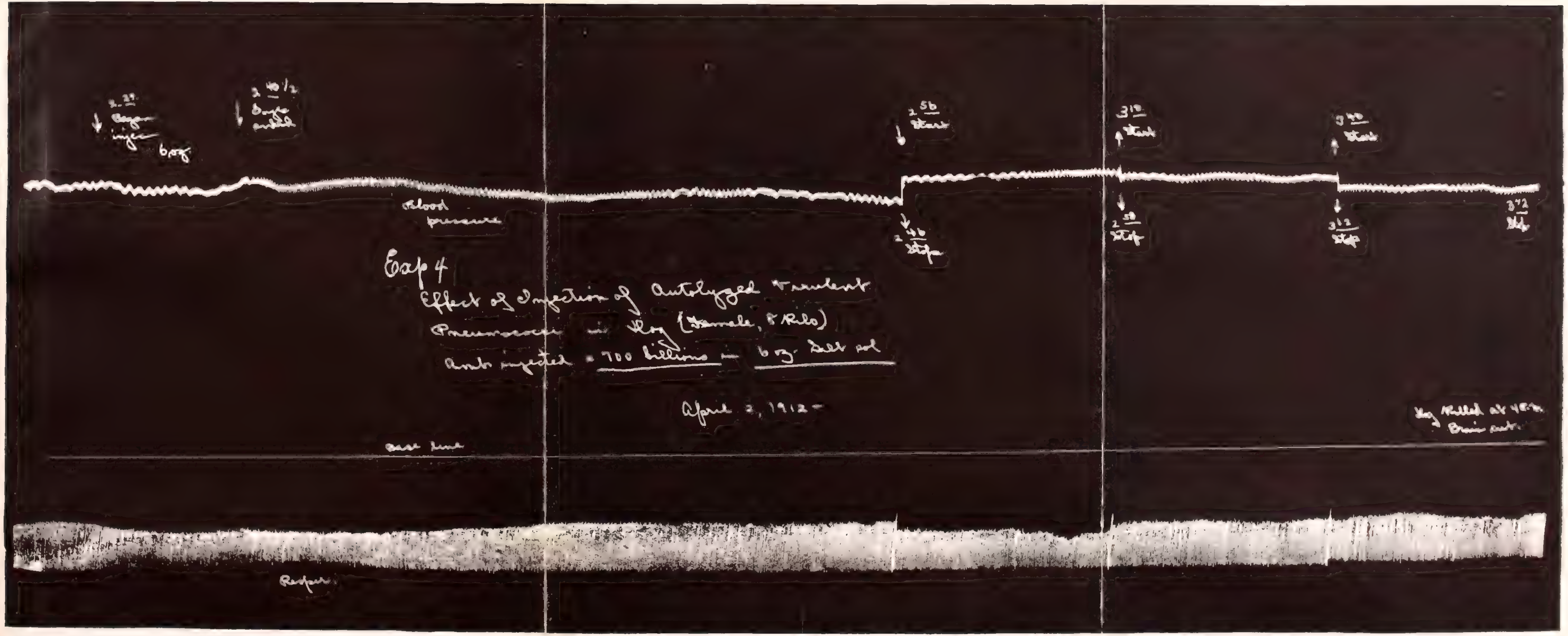
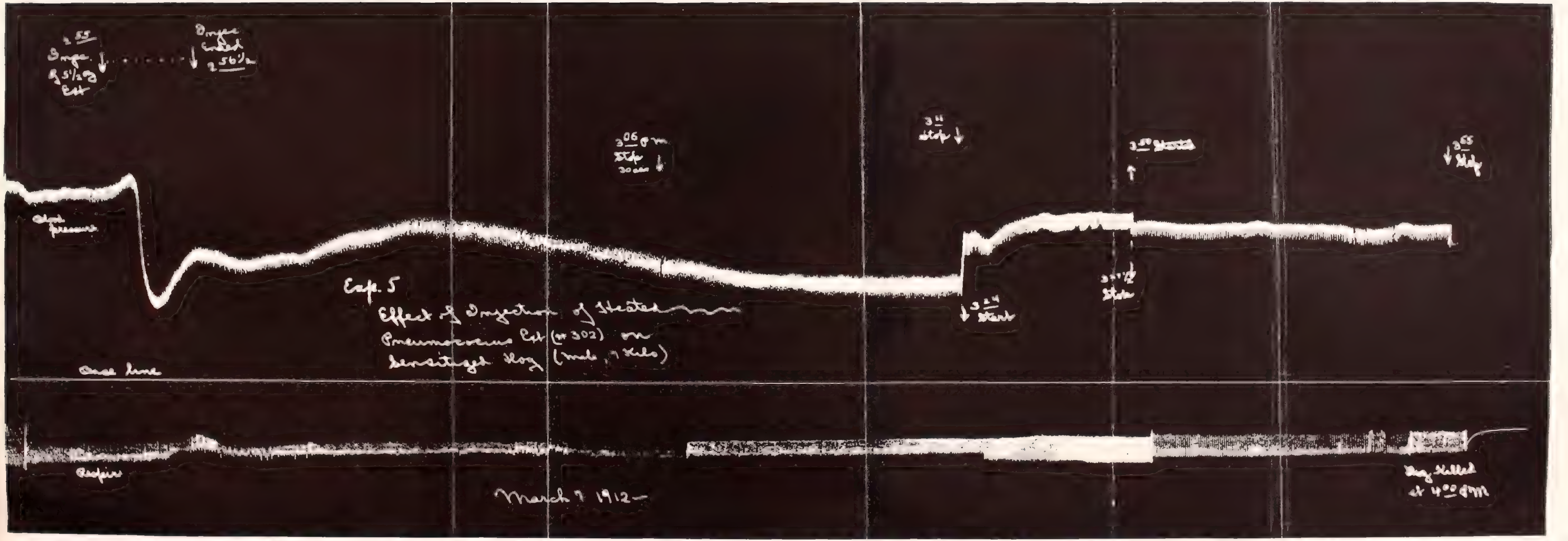

Exp. 3
Effect of Injection of
Virulent Pneumococcus
in Dog intravenously
(1 cc of Suspension from 800
c.c. of broth culture)
Dog. male

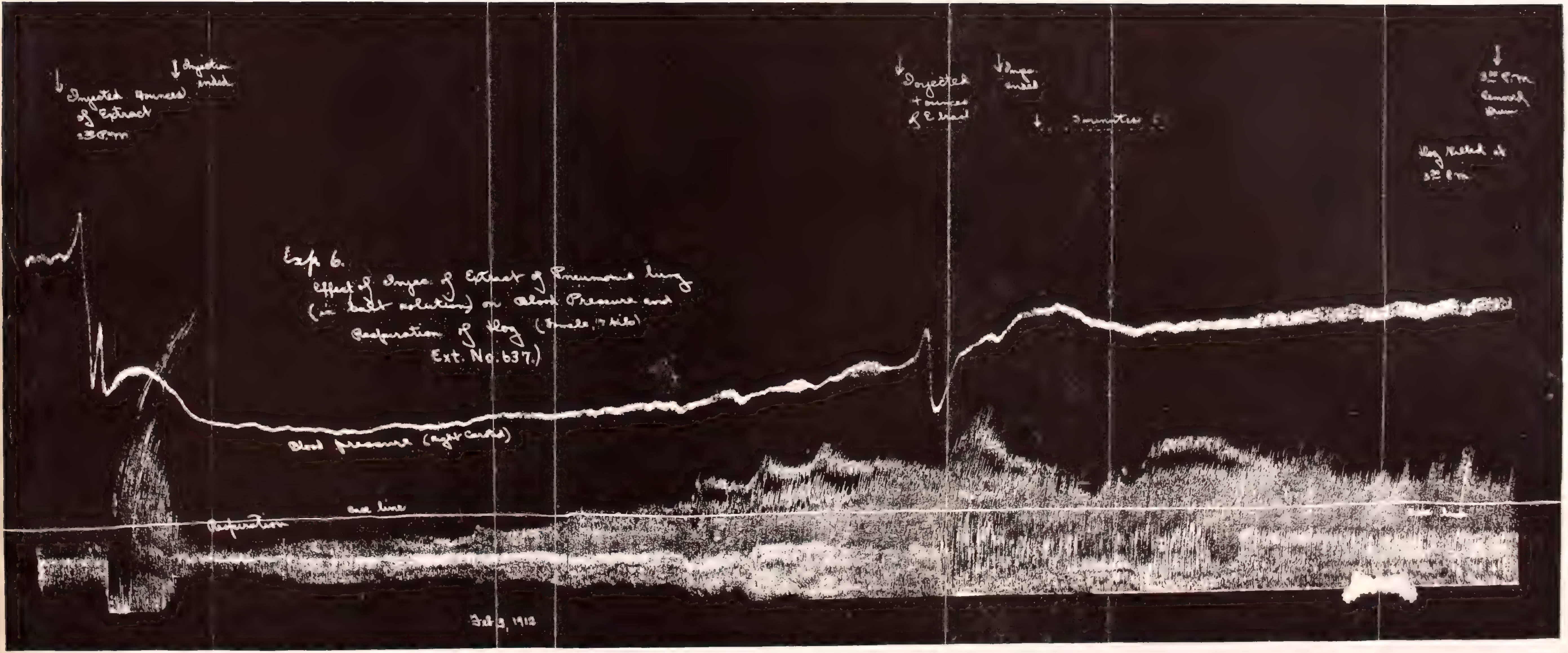
PLATE 7.

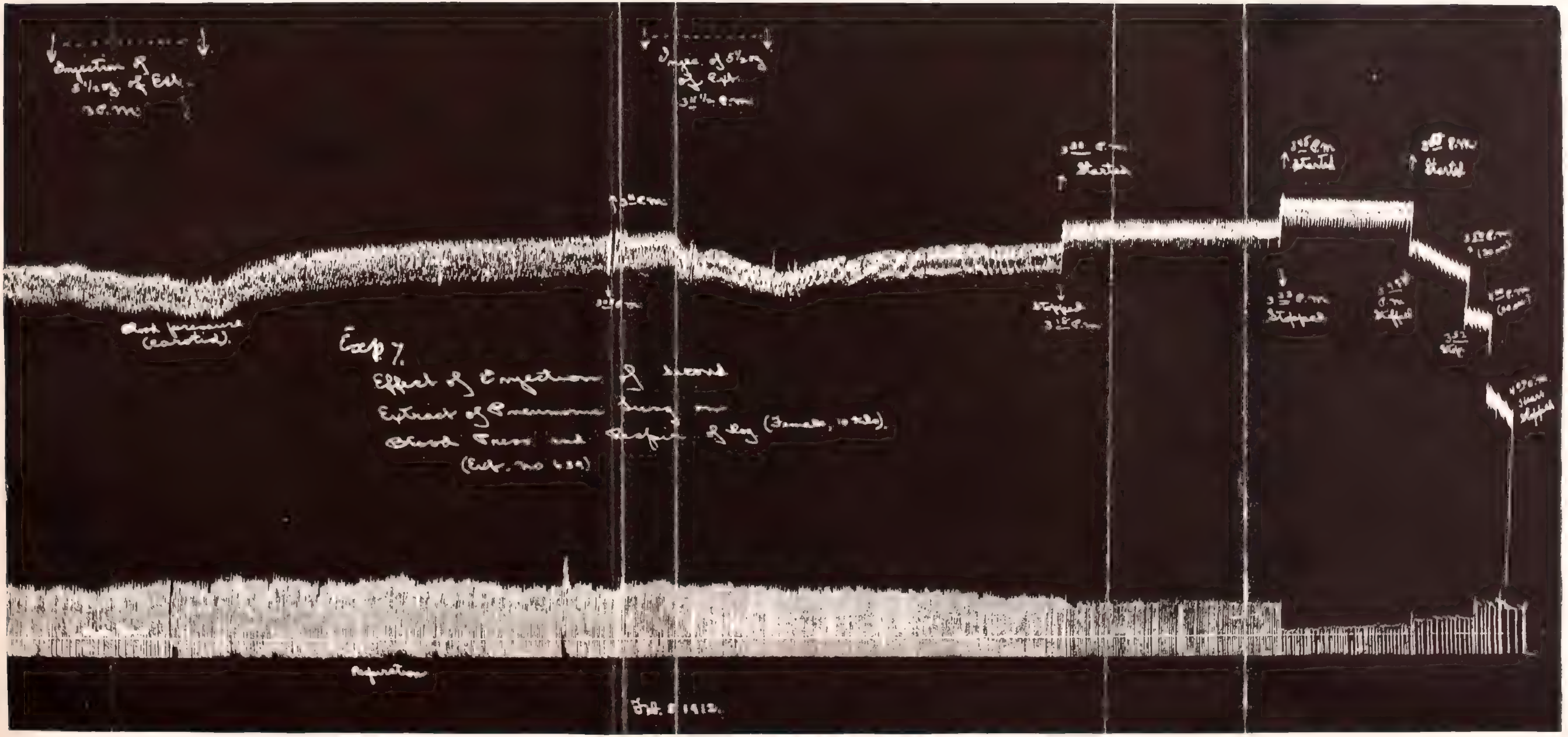


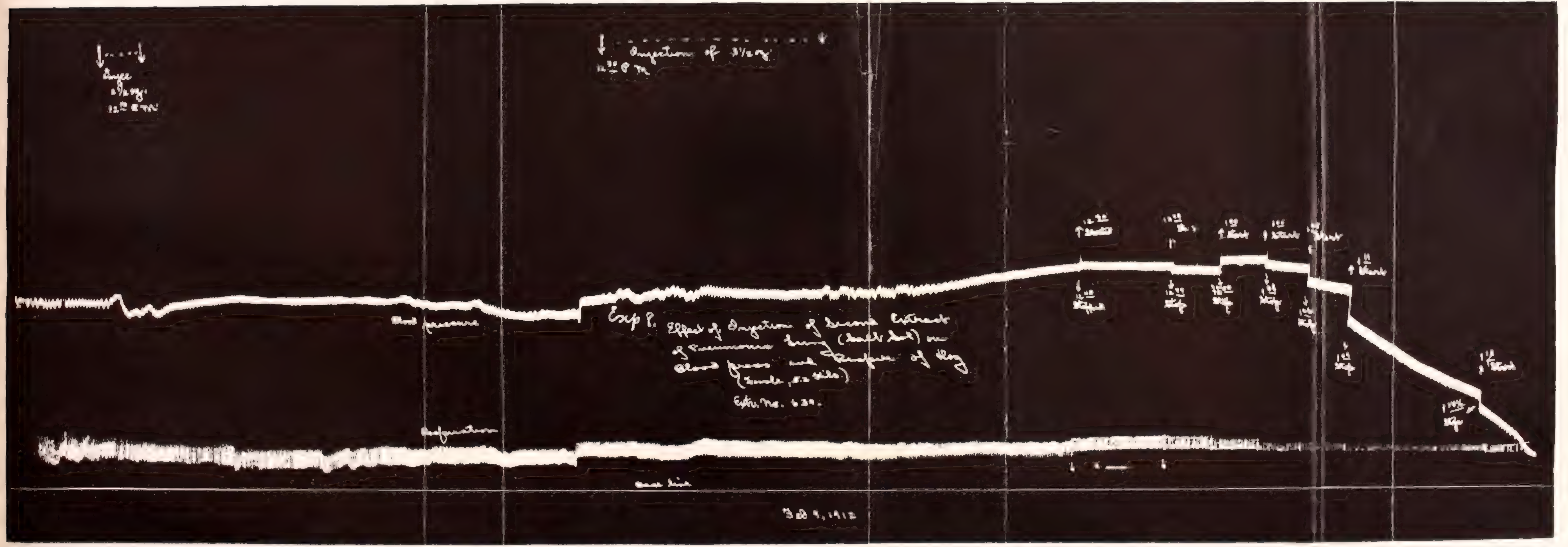
Exp 3 - Continued
Blood press
1 minute
Dog killed at 6 P.M.
(No 2)
March 15, 1912


Blood pressure
Exp 4
Effect of Injection of Autolyzed Virulent
Pneumococci in Dog (Female, 8 Kilo)
April 2, 1912


Blood pressure
Exp. 5
Effect of Injection of Heated
Pneumococcus Ext (# 302) on
Sensitized Dog (male, 7 Kilo)
Base line
Respir
March 7 1912
Dog killed at 4 P.M.


Exp 6.
Effect of Injec. of Extract of Pneumonic lung
(in salt solution) on Blood Pressure and
Respiration of Dog
Ext. No. 637.)
Injected 4 ounces of Extract
Injection ended
Blood pressure
Respiration
Feb 3, 1912


Exp 7.


Exp 8. Effect of Injection of

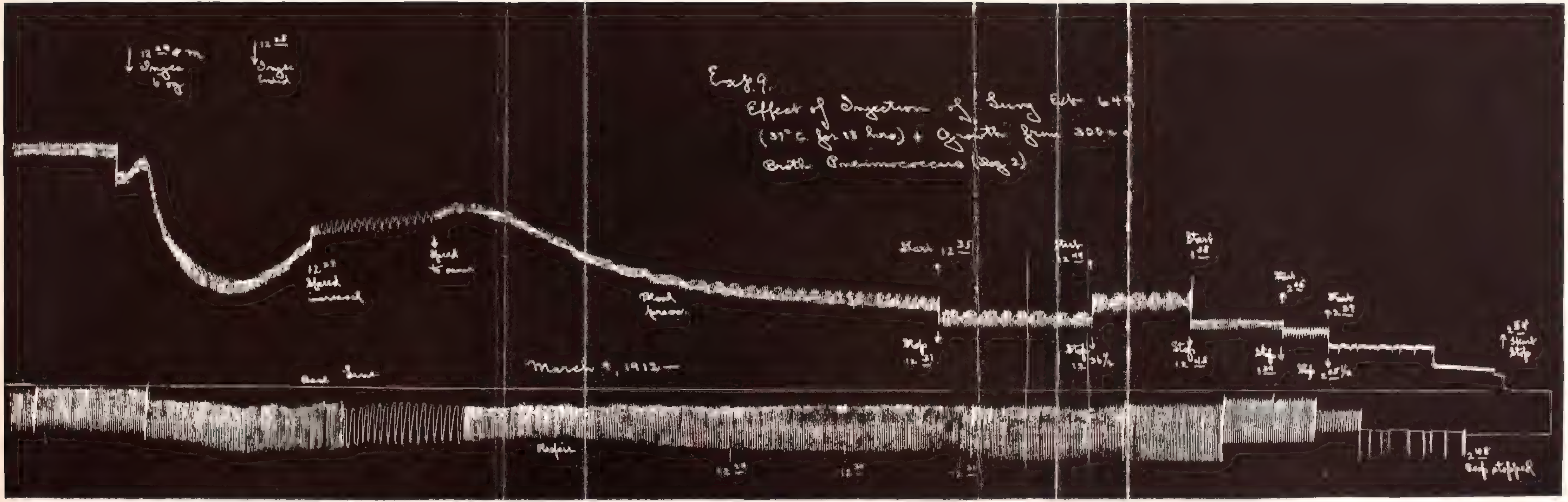
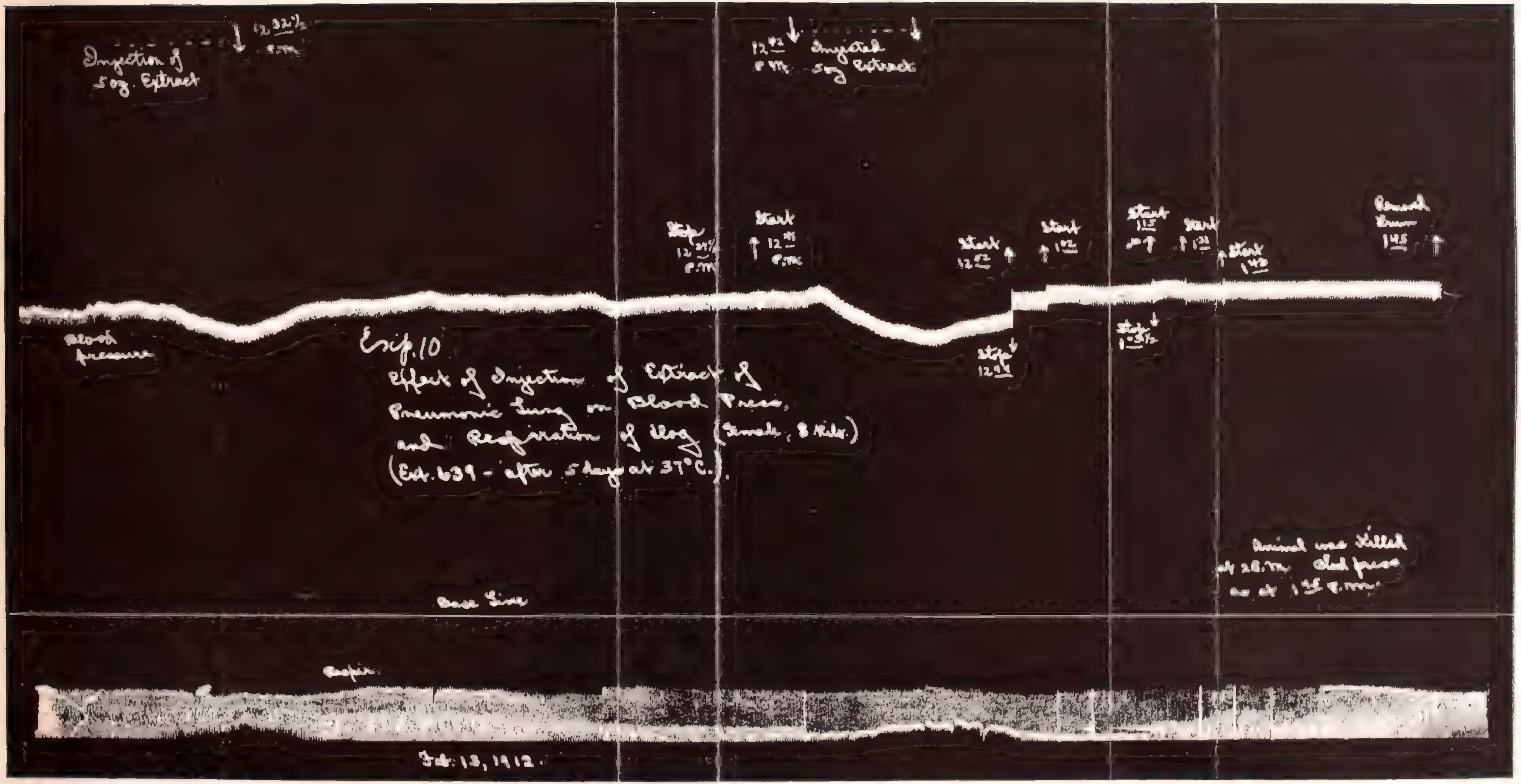

Exp. 9.
Effect of Injection of Lung Ext 649
(37° C. for 18 hrs) + Growth from 300 c.c.
Broth Pneumococcus (Day 2)
March 9, 1912—
Start 12 35
Start 1 08
Repair

PLATE 14.


Injection of .5 oz. Extract
Injected .5 oz Extract
Stop
Start
Start
Start
Start
Start
Start
Stop
Stop
Blood pressure
Exp. 10
Effect of Injection of Extract of
Pneumonic Lung on Blood Press.
and Respiration of Dog (Female, 8 Kilo.)
(Ext. 639 - after 5 days at 37°C.)
Animal was killed
Base Line
Expir.
Feb. 13, 1912.

GENERAL INDEX

SUBJECT INDEX.

INDEX OF AUTHORS.

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